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AMINO ACID RESIDUES ESSENTIAL FOR CATALYSIS BY PEPTIDYL DIPEPTIDASE-4 FROM PSEUDOMONAS MALTOPHILIA

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To assess residues essential for catalysis by prokaryotic peptidyl dipeptidase-4, the enzyme was subjected to chemical modification by a series of reagents. Treatment with either tetranitromethane or N-acetylimidazole abolished catalytic activity. Hydroxylamine reversed inactivation by acetylimidazole only. Thus, an essential tyrosine is indicated. Enzymatic activity also was quenched by either trinitrobenzenesulfonic acid or diethyl pyrocarbonate. Inactivation by these reagents was not reversed by hydroxylamine. These data suggest an essential lysine. The competitive inhibitor Phe-Arg protected partially against inactivation by tetranitromethane, and fully against inactivation by N-acetylimidazole. The substrate Hip-Phe-Arg protected against inactivation by trinitrobenzenesulfonic acid and diethyl pyrocarbonate. Thus, both tyrosine and lysine are located at the catalytic site.

In hospitalized patients, gram-negative non-fermentative bacteria are an important cause of infection (1). Pseudomonas maltophilia is the second most common Pseudomonad isolated from clinical specimens (2), and has been implicated as a cause of pneumonia in compromised hosts (3). Peptidyl dipeptidase-4 (PDP-4) from P. maltophilia hydrolyzes peptide substrates by releasing dipeptide units from the carboxy terminus. In this manner, it converts angiotensin-1 to angiotensin-2, inactivates bradykinin, and converts atriopeptin-2 to atriopeptin-1 (4). Although the role of PDP-4 in P. maltophilia remains to be established, through the synergistic hydrolysis of vasoactive peptides PDP-4 may assist the margination of P. maltophilia from blood to

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Abbreviations: PDP-4, peptidyl dipeptidase-4; ACE, angiotensin converting enzyme; DEAE, diethyl aminoethyl; Hip, hippuryl; TFA, trifluoroacetic acid; TNM, tetranitromethane; DEPC, diethyl pyrocarbonate; BSA, bovine serum albumin.

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tissues in susceptible hosts. Alternately, PDP-4 may contribute to the necrotizing lung injury of pneumonia. Woods et al have implicated Pseudomonad exoenzymes as determinants of virulence in lung injury (5,6).

Recently, we identified an essential Arg in PDP-4 (7). In this manuscript, we extend chemical modification studies with PDP-4 to assess amino acids other than Arg which may be essential for catalysis by PDP-4 and to determine if PDP-4 has essential amino acids common to other enzymes which function wholly or in part as carboxy-terminal exopeptidases.

MATERIALS AND METHODS

PDP-4 was purified to near homogeneity by a series of chromatographic steps (manuscript submitted). Briefly, a supernatant fraction from P. maltophilia extracellular medium was subjected to DEAE-cellulose chromatography followed by DEAE-Sephacel, hydroxylapatite, copper metal chelate affinity, and Ultrogel AcA-34 size exclusion chromatographies respectively. After these steps, PDP-4 was assessed to be 95% pure by sodium dodecyl sulfate polyacrylamide gel electrophoresis, and had a specific activity of 101 μmol/min/mg against 5mM Hip-His-Leu in 0.1M HEPES, pH 8.0, 20μM lisinopril at 37°C.

Chemical modifications were done at 25°C with solutions of 5nM PDP-4 containing 50 μg/ml BSA to stabilize PDP-4. Dilute solutions of PDP-4 lost catalytic activity rapidly unless stabilized with BSA ballast protein. With BSA, all PDP-4 controls remained fully active over the time period for each experiment. Concentrated reagent solutions were prepared as follows: diethyl pyrocarbonate, N-acetylimidazole, and N-ethylmaleimide in 50mM HEPES, pH 7.5; tetranitromethane in 95% ethanol; trinitrobenzenesulfonic acid in 100mM potassium phosphate, pH 8.5; hydroxylamine.HCl in 50mM HEPES, adjusted to pH 7.1 with saturated potassium hydroxide. Reagents were diluted into solutions of PDP-4 in the same buffer used for each reagent except for tetranitromethane which was added to PDP-4 in 50mM Tris, pH 8.0.

Residual enzymatic activity was determined at 37°C for 30 min with 10 μl sample in 100 μl of 5.5mM Hip-His-Leu substrate in 0.1M HEPES, pH 8.0. All samples modified in the presence of Phe-Arg or Hip-Phe-Arg, along with appropriate controls, were dialyzed for 24h at 5°C before being assayed. Enzymatic activity was quenched with 5μl 50% trifluoroacetic acid (TFA). The product of hydrolysis, hippuric acid, was quantitated by high performance liquid chromatography after separation of 50 μl aliquots on a Brownlee Aquapore RP-300 column as described previously (8), except that the concentration of the ion-pairing agent, TFA, was 0.05%.

Hip-His-Leu, Hip-Phe-Arg and Phe-Arg were from Bachem, Inc, Torrance, CA. Tetranitromethane, N-acetylimidazole, diethyl pyrocarbonate, and hydroxylamine HCl were from Aldrich Chemical Co, Milwaukee, WI. 2,4,6-trinitrobenzenesulfonic acid and N-ethylmaleimide were from Eastman Kodak, Rochester, NY.

RESULTS

Upon treatment with 5mM N-acetylimidazole, prepared immediately before use, PDP-4 activity was reduced to less than 15% relative to an untreated

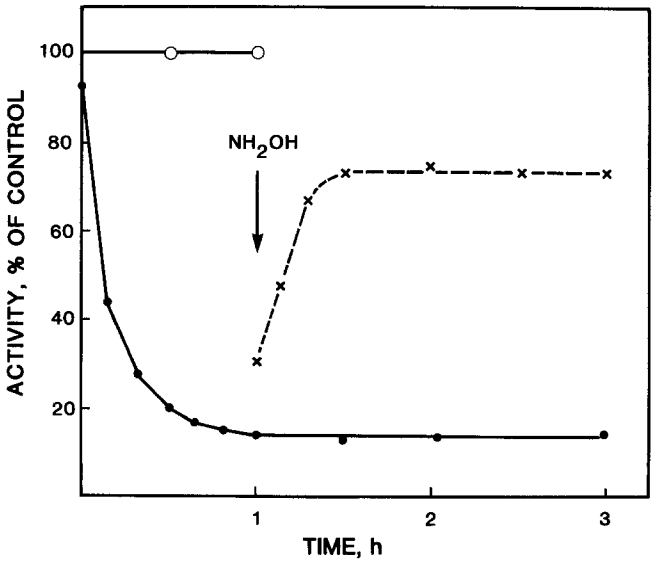

Fig 1. Treatment of PDP-4 with 5mM N-acetylimidazole (•). After 1h an aliquot was removed and treated with hydroxylamine (x). Sample modified in the presence of 10mM Phe-Arg (o).

control (Fig 1). After 1h in the presence of acetylimidazole, the modified PDP-4 was treated with 0.25M hydroxylamine.HCl whereupon activity increased to 73% of control within 30 min and remained constant for up to 2h. Dialysis for 24h did not reverse PDP-4 inactivation by acetylimidazole or any other modifying reagent discussed in this manuscript. When PDP-4 was treated with acetylimidazole in the presence of 10mM Phe-Arg, a competitive inhibitor (K_i, 14μM), inactivation was blocked. Acetylimidazole modifies both Cys and Tyr, and rarely under some conditions Lys (9). However, deacetylation with hydroxylamine suggests that Tyr was modified.

To confirm Tyr modification, PDP-4 was treated with 3mM tetranitromethane at pH 8.0. Activity was reduced to less than 15% within 10 min. With 10mM Phe-Arg in the sample, 64% activity remained after 30 min (Fig 2). At pH 8, tetranitromethane nitrates Tyr and oxidizes Cys; at pH 6, only Cys oxidation occurs (10). When PDP-4 was treated with tetranitromethane in 100mM potassium phosphate, pH 6.0, 71% activity was recovered after 30 min.

PDP-4 is not inactivated by the Cys reagent N-ethylmaleimide (8). When PDP-4 was treated with 2.5mM N-ethylmaleimide, pH 7.5, for 1h, activity remained at 99%. Subsequent treatment with tetranitromethane, either before

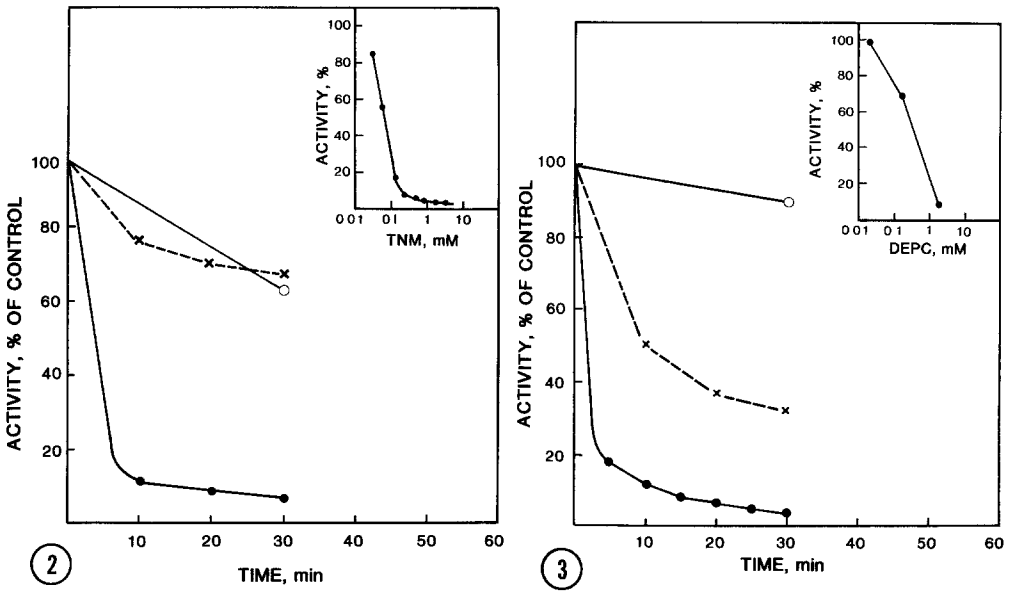

Fig 2. Time course of PDP-4 inactivation with 3mM tetranitromethane in the presence, pH 8 (o), and absence of 10mM Phe-Arg, pH 8 (•) and pH 6 (x). Insert: Effect of TNM concentration on PDP-4 activity after 30 min at 25°C.

Fig 3. Time course of PDP-4 inactivation with 2mM diethyl pyrocarbonate in the presence, pH 7.5 (o), and absence of 1mM Hip-Phe-Arg, pH 7.5 (•) and pH 6 (x). Insert: Effect of DEPC concentration on PDP-4 activity after 30 min at 25°C.

or after dialysis to remove excess N-ethylmaleimide, reduced activity to 4% within 30 min. Thus, blocking Cys does not protect against inactivation by tetranitromethane which would be specific for Tyr under these conditions.

Modification of PDP-4 with 2mM diethyl pyrocarbonate, pH 7.5, reduced activity to 5% within 30 min (Fig 3). Inactivation was not reversible by 0.5M hydroxylamine.HCl within 1h. Both 1mM Phe-Arg and 1mM Hip-Phe-Arg, a substrate, protected against inactivation by diethyl pyrocarbonate. Diethyl pyrocarbonate is attacked by the unprotonated nucleophilic forms of His and Lys (11). Therefore, at pH 6 the reagent should be more selective for His. When modification was done in 100mM potassium phosphate, pH 6.0, activity was reduced to only 32% within 30 min and was not reversible with hydroxylamine. Hydroxylamine removes the carbethoxy group from mono-modified His but not Lys. However, with excess reagent His could be modified at both nitrogens, a reaction that is not reversed by hydroxylamine (12). Therefore, for these

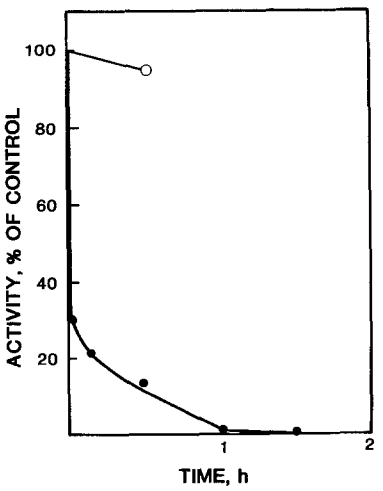

Fig 4. Treatment of PDP-4 with 5mM trinitrobenzenesulfonic acid in the presence (o) and absence (•) of 1mM Hip-Phe-Arg.

experiments we used the lowest amount of reagent which would inactivate PDP-4 within the half-life of the labile reagent. The data support the conclusion that Lys was modified.

To confirm an essential Lys, PDP-4 was treated with 5mM trinitrobenzenesulfonic acid. Activity was reduced to 30% within 1 min and was undetectable after 60 min (Fig 4). 1mM Hip-Phe-Arg protected against inactivation, as did 1mM Phe-Arg.

DISCUSSION

In its action upon substrates, PDP-4 is similar to the better known angiotensin converting enzyme (ACE, EC 3.4.15.1) (13), an enzyme that has been studied extensively because of its key role in both the renin-angiotensin and kallikrein-kinin systems. Both enzymes display broad specificity with peptide substrates (14, manuscript submitted), yet distinct differences among peptides susceptible to hydrolysis have been documented. For example, substance P is hydrolyzed by ACE but not by PDP-4 (4,15); whereas atriopeptin-2 is hydrolyzed by PDP-4 but not by ACE (4,8). Furthermore, lisinopril is a potent ACE inhibitor but does not inhibit PDP-4 (8,16).

To elucidate factors contributing to the molecular mechanism of substrate hydrolysis, we have analyzed PDP-4 by chemical modification of amino acids. Treatment of PDP-4 with acetylimidazole eliminated catalytic activity. Deacetylation by hydroxylamine with concomitant recovery of activity indicated that Tyr had been modified. This was confirmed by modification with tetranitromethane which eliminated catalytic activity also. Protection against inactivation by the competitive inhibitor Phe-Arg suggested that Tyr was located at or near the PDP-4 active site. Similarly, an essential Tyr has been identified at the active site of ACE (17,18). It has been hypothesized that Tyr in ACE donates a proton to the nitrogen of the scissile peptide bond which is positioned correctly through interaction of the substrates' terminal carboxyl group with an active site Arg (19). Our previous finding that PDP-4 contains essential Arg (7) suggests that Tyr and Arg in PDP-4 may function like these residues in ACE. It is likely that such interactions are relevant to metalloexopeptidases in general, because Arg-145 and Tyr-248 in carboxypeptidase A are believed to mimic functionally their counterparts in ACE (19).

In addition to Tyr, our experiments identified a functional Lys in PDP-4. Upon modification with diethyl pyrocarbonate or trinitrobenzenesulfonic acid, PDP-4 activity was quenched. Activity was not restored by hydroxylamine. However, Phe-Arg prevented inactivation by either reagent. In principle, Phe-Arg reacts with amino-selective reagents and could have protected against inactivation by scavenging the modifying reagents. To establish that protection by Phe-Arg did not occur because the Lys modifying reagents were consumed, the reagents were used at concentrations at least 2-fold greater than Phe-Arg. Furthermore, the substrate Hip-Phe-Arg, which does not react with the reagents, protected against inactivation under conditions where less than 10% of the substrate was hydrolyzed. These data suggested that the essential Lys was associated with the PDP-4 active site. Essential Lys have been identified in ACE (17,18,20,21), one of which is presumed to be the site for anion binding (22). Others may play a more direct role in substrate hydrolysis (21). Since PDP-4 lacks an anion requirement for activity, Lys in PDP-4

cannot function solely as it does in ACE. The role of Lys in PDP-4 catalysis remains to be elucidated. (Supported by NHLBI Research Grants HL14456 and HL07053.)

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