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DETECTION OF ESSENTIAL ARGININE IN BACTERIAL PEPTIDYL DIPEPTIDASE-4: ARGININE IS NOT THE ANION BINDING SITE

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Peptidyl dipeptidase-4 from Pseudomonas maltophilia was modified with the arginine reagents p-hydroxyphenylglyoxal and 2,3-butanedione. The enzyme was inactivated in a pseudo-first-order manner by p-hydroxyphenylglyoxal with a half-time of 72 min. Inactivation by 2,3-butanedione was biphasic with a rapid phase followed by a slower inactivation to less than 10% activity within 24h. The competitive inhibitor thiorphan protected against inactivation by p-hydroxyphenylglyoxal and by 2,3-butanedione also but to a lesser degree. Inhibitory anions chloride and phosphate did not protect against inactivation by either reagent. These data support the conclusion that an active site arginine is essential for substrate hydrolysis. Furthermore, arginine is not the binding site for the inhibitors chloride and phosphate.

Metalloenzymes with carboxy terminal exopeptidase activity, including carboxypeptidases A and B (EC 3.4.17.1 and EC 3.4.17.2)(1), angiotensin converting enzyme (EC 3.4.15.1)(2), and neutral metalloendopeptidase (EC 3.4.24.11)(3,4) have an Arg residue which is essential for hydrolysis of peptide substrates. Conversely, thermolysin (EC 3.4.24.4), which functions primarily as an endopeptidase, lacks an essential Arg (4). Thus, near neutral pH exopeptidase activity presumes a positively charged Arg in or near the catalytic site juxtapositioned to interact with the negative charge at the

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Abbreviations: PDP-4, peptidyl dipeptidase-4; ACE, angiotensin-converting enzyme; NEP, neutral metalloendopeptidase; DEAE, diethyl aminoethyl; Hip, hippuryl; TFA, trifluoroacetic acid; SP, substance P; BD, 2,3-butanedione; PG, p-hydroxyphenylglyoxal; BSA, bovine serum albumin.

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carboxy terminus of a peptide substrate. In addition, Williams and Auld suggest that Arg in carboxypeptidase A is the binding site for the inhibitory anions chloride and phosphate (5).

Pseudomonas maltophilia secretes a peptidyl dipeptidase, bacterial PDP-4, which hydrolyzes a broad spectrum of substrates including the vasoactive peptides angiotensin-1, bradykinin, and atriopeptin-2 (6). Thus, in a nosocomial setting, compromised hosts susceptible to P. maltophilia infection (7) may be subjected to hypertensive effects induced by the synergistic actions of bacterial PDP-4 mediated generation of angiotensin-2, destruction of bradykinin, and modulation of atriopeptin activities.

To some degree, PDP-4 catalytic activity resembles that of ACE. However, unlike ACE, PDP-4 has no anion requirement for activity. To the contrary, PDP-4 is inhibited by anions (6), and in this regard resembles carboxypeptidase A (5).

Recently, we determined that residues essential for PDP-4 catalysis include Tyr and Lys (8). To determine if PDP-4 contains functional Arg, we treated the enzyme with both p-hydroxyphenylglyoxal (9) and 2,3-butanedione (1).

MATERIALS AND METHODS

PDP-4 was purified to near homogeneity by a series of chromatographic steps (6). 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 101umol/min/mg against 5mM Hip-His-Leu in 0.1M HEPES, pH 8.0, 20uM lisinopril at 37°C.

Chemical modifications were done at 25°C with solutions of 5nM PDP-4 containing 50ug/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.

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

Hip-His-Leu was from Bachem, Inc., Torrance, CA. 2,3-butanedione was from Aldrich Chemical Co., Milwaukee WI. p-hydroxyphenylglyoxal was from Pierce Chemical Co., Rockford, IL. Thiorphan was a gift from Dr. D.W. Cushman, Squibb Institute, New Brunswick, NY.

RESULTS

Bacterial PDP-4 catalytic activity was decreased upon modification with p-hydroxyphenylglyoxal in a time and concentration dependent manner (Fig 1). With 12mM reagent, loss of activity was a pseudo-first-order process with a half-time of inactivation of 72 min and was not reversible upon dialysis. The rate of inactivation was not altered significantly when modification was done in the presence of the inhibitory anions chloride and phosphate (Table 1). However, the competitive inhibitor thiorphan (K_i, 1.2um)(6) protected the enzyme against inactivation. These data suggest that Arg is essential for catalytic activity and that at least one Arg is at the active site.

To insure that p-hydroxyphenylglyoxal inactivation did not result from modification of alpha-amino residues, a second modification was done with 2,3-butanedione. The presence of essential Arg was confirmed (Fig 2). The time course for inactivation of PDP-4 was concentration dependent, decreasing as the concentration of butanedione increased. Inactivation occurred rapidly during the first 30-60 min, and thereafter more slowly over a period of several hours. When chemical modification was done in borate buffer, rather than HEPES buffer, the rate of inactivation at 25°C was enhanced. The rate of inactivation was not altered in the presence of either 1M sodium chloride or 1M potassium phosphate (Table 1). After 24h in borate, less than 10% activity was present relative to unmodified control PDP-4 which remained fully active during the experiment. Upon dialysis overnight at 5°C against 20mM potassium phosphate, pH 8.3, inactivation was partially reversible (ie, 35% of control) when dialysis was commenced after 3h in the presence of 10mM butanedione, but was not reversible when commenced after 24h.

To determine if modification alters the substrate specificity of PDP-4, hydrolysis of substance P (SP), SP free acid, and SP methyl ester were compared for the modified and unmodified enzymes. PDP-4 has an absolute require-

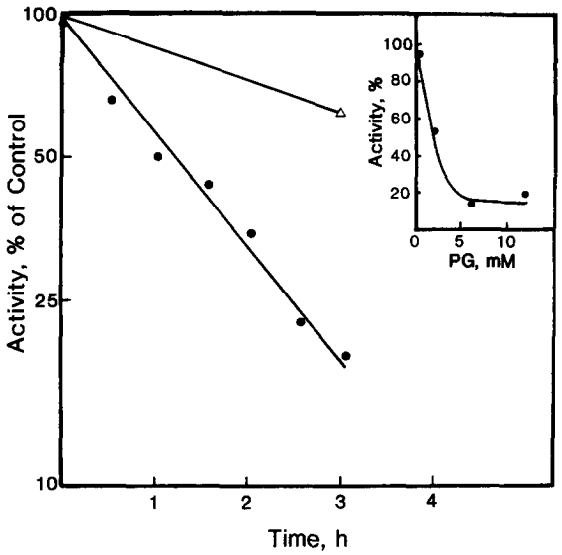

Fig 1. Treatment of bacterial PDP-4 with p-hydroxyphenylglyoxal (PG). PDP-4 was incubated with 12mM PG in 0.2M N-ethylmorpholine, pH 8.0, in the presence (Δ) and absence (•) of 5mM thiorphan at 25°C. Each value is the mean of 2-4 determinations. Insert: effect of PG concentration on PDP-4 activity after 3h at 25°C.

ment for substrates with a free carboxy-terminus (6). Thus, SP free acid is hydrolyzed by the unmodified enzyme, but SP (with a carboxy-terminal amide group) and SP methyl ester are not. Butanedione modification of PDP-4 did not alter the substrate specificity of the enzyme. Modified PDP-4 hydrolyzed only SP free acid when activity was reduced 50% by butanedione. None of the substrates were hydrolyzed when activity was reduced to virtually zero.

Table 1

Effect of inhibitors upon modification of bacterial PDP-4

Inhibitor	12mM p-hydroxyphenylglyoxal, half-time (min)	10mM 2,3-butanedione, residual activity (% of control)
none	72	24
sodium chloride, 1M	66	18
potassium phosphate, 1M	84	20
thiorphan‡	264	66

Activities were determined after 3h, except for thiorphan containing samples where overnight dialysis was commenced after 3h and residual activity was determined after dialysis. Each value is the mean of 2-3 determinations. BD modification was done in 50mM sodium borate, pH 7.6.
‡ Thiorphan concentrations were 5mM with PG and 10mM with BD.

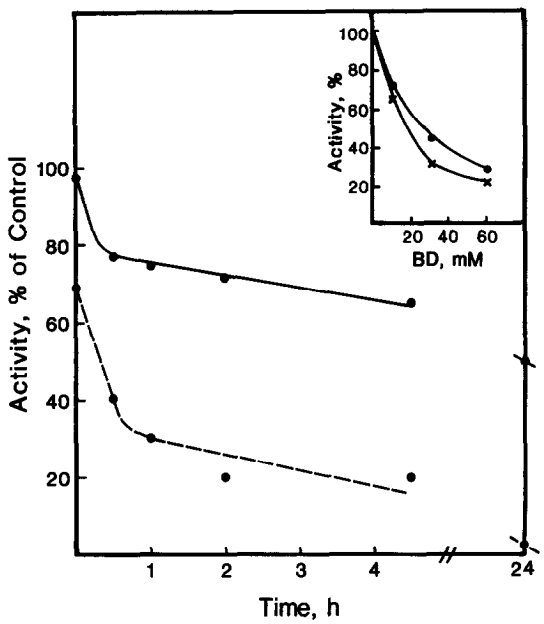

Fig 2. Time course of bacterial PDP-4 inactivation with 10mM 2,3-butanedione (BD) in either 0.1M HEPES, pH 8.0 (——) or 50mM sodium borate, pH 7.6 (---). Insert: effect of BD concentration on PDP-4 activity after 2h (•) and 3h (x) at 25°C in borate buffer.

Therefore, fully modified PDP-4 does not acquire the ability to hydrolyze substrate structures not susceptible to hydrolysis by the unmodified enzyme.

DISCUSSION

Bacterial PDP-4 was inactivated by the relatively specific Arg reagent p-hydroxyphenylglyoxal (9) in a pseudo-first-order reaction. Essential Arg was confirmed with 2,3-butanedione which, as used here, is specific for the modification of Arg residues in proteins (1,12). Upon treatment of PDP-4 with butanedione in borate buffer, peptide hydrolyzing activity was reduced 70% within 1h and subsequently more slowly to less than 10% within 24h. Enhancement of inactivation by borate is characteristic of Arg modification by butanedione (1). The biphasic response with butanedione may reflect differential modification of Arg populations in distinct microenvironments, or modification followed by conformational perturbation. These data indicate that PDP-4 contains one or more Arg residues essential for catalytic activity.

Protection against inactivation was observed with thiorphan added prior to, and present during, incubation with p-hydroxyphenylglyoxal. Thiorphan protection against butanedione was less extensive. This latter observation is analogous to that reported by Riordan with carboxypeptidase A, where the active site Arg was not protected fully against butanedione inactivation by the competitive inhibitor beta-phenyl propionate (1). With carboxypeptidase A, both chloride and phosphate protect againt inactivation by butanedione (5) leading Williams and Auld to conclude that Arg is the binding site for inhibitory anions. However, neither chloride nor phosphate protected bacterial PDP-4 against inactivation by Arg reagents. Thus, it is unlikely that Arg is the PDP-4 anion binding site for either of these inhibitory anions.

It has been recognized that exopeptidases with specificity for peptide bonds at or near the carboxy terminus of a substrate have residues at their catalytic clefts that contribute a complimentary positively charged binding site which interacts through ionic bonding with the negatively charged carboxy terminus thereby positioning the scissile bond at the appropriate locus for hydrolysis. This residue has been identified as Arg for carboxypeptidases (13-15). Presumably the essential Arg of NEP (4,16) and ACE (17) serve an analogous function. However, thermolysin, which functions primarily as an endopeptidase (18,19), has an active site Arg which is not essential for catalytic activity. Upon modification with butanedione, thermolysin activity is increased rather than decreased (4). Kester and Matthews have suggested that this Arg interacts with the carbonyl group of the P_1' residue rather than the carboxy terminus (19). It has been hypothesized that zinc metalloexopeptidases, as a class, have evolved divergently with retention of some common active site features even though they are quite different structurally (20). Conversely, thermolysin, which may resemble the others in part, may have acquired those features through convergent evolution (14).

Although the metal ion of the metalloexopeptidase PDP-4 remains unknown to date, it appears that PDP-4, like several other metalloexopeptidases, requires Arg for catalytic activity. These data suggest that this essential Arg may

function like those of ACE and NEP, rather than the non-essential Arg in thermolysin.

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