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IDENTIFICATION OF ESSENTIAL AMINO ACID RESIDUES IN CLOSTRIDIUM HISTOLYTICUM COLLAGENASE USING CHEMICAL MODIFICATION REACTIONS

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Summary: Clostridium histolyticum collagenase has been chemically modified with a series of reagents to identify essential amino acid residues. The activity of the enzyme is not significantly altered by the seryl reagents diisopropylfluorophosphate and phenylmethylsulfonyl fluoride, the cysteinyl reagents p-chloromercuribenzoate and iodoacetamide, or the arginyl reagents butanedione and phenylglyoxal. The enzyme is inactivated by 1-ethyl-3(3-dimethylaminopropyl)-carbodiimide and N-ethyl-5-phenylisoxazolium-3'-sulfonate, indicating the presence of an essential carboxyl residue. Both acetylimidazole and tetranitromethane inactivate the enzyme and the acetylimidazole reaction is reversed by hydroxylamine, indicating that collagenase contains an essential tyrosyl residue. In addition, acylation of the enzyme by diethylpyrocarbonate, diketene and acetic anhydride markedly lowers activity, which cannot be restored by hydroxylamine. This indicates that collagenase contains an essential lysyl residue, a conclusion supported by the fact that trinitrobenzene sulfonate also inactivates the enzyme.

Introduction: The most widely studied collagenases are those from the bacterium Clostridium histolyticum (EC 3.4.24.3). These enzymes, which are members of a broad class of neutral metalloproteinases that contain zinc at the active site, are unique in their ability to hydrolyze the triple-helical region of collagen under physiological conditions (1). Apart from the catalytic metal, very little attention has been directed toward identifying other functional constituents of the active site (2,3). We have purified the major collagenase from Clostridium histolyticum and have examined the effects of a series of chemical modification

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reactions on the activity of the enzyme in order to establish which amino acid residues are essential for activity.

Materials and Methods: Crude collagenase was obtained from Sigma Chemical Co. (Type II) and the major collagenase present was purified to homogeneity (4). This enzyme is devoid of activity toward both benzoyl-Arg-O-CH_2CH_3 and [$^{14}CH_3$]-casein and has a specific activity toward acid soluble [$^{14}CH_3$]-collagen (calf skin) of 1.3 pkat/μg when assayed in the presence of thermolysin (5). The activity of the enzyme was measured using 2-furanacryloyl-Leu-Gly-Pro-Pro (FALGPP) as substrate, as described earlier (6). Initial rates were measured spectrophotometrically by monitoring the decrease in absorbance at 324 nm of 0.05 mM FALGPP in 50 mM N-tris(hydroxymethyl)methylglycine (Tricine), 0.4 M NaCl, 10 mM $CaCl_2$, pH 7.5. Diisopropylfluorophosphate, iodoacetamide, butanedione, tetranitromethane, diketene, acetylimidazole, diethylpyrocarbonate, and N-ethyl-5-phenylisoxazolium-3'-sulfonate (Woodwards Reagent K) were purchased from Aldrich Chemical Co., phenylmethylsulfonyl fluoride, p-chloromercuribenzoate, trinitrobenzene sulfonate and 1-ethyl-3(3-dimethylaminopropyl)-carbodiimide (EDC) from Sigma Chemical Co., phenylglyoxal from Pfaltz and Bauer and acetic anhydride from Mallinckrodt Chemical Co. Carbobenzoxy-Gly-Pro-Gly-Gly-Pro-Ala (Cbz-GPGGPA) was a product of Sigma Chemical Co. All reactions were carried out at 22°C in either borate, N-2-hydroxyethylpiperazine-N'-2-ethane sulfonate (Hepes), 2-(N-morpholino)ethane sulfonate (Mes), or tris(hydroxymethyl)-aminomethane (Tris) buffer containing 10 mM $CaCl_2$.

Results: Collagenase is completely inhibited by a 1 mM concentration of the chelating agents 1,10-phenanthroline and ethylenediaminetetraacetic acid (Table I). This is consistent with the view that collagenase is a metalloenzyme. In contrast, its activity is essentially unaltered by the serine proteinase inhibitors diisopropylfluorophosphate and phenylmethylsulfonyl fluoride and the cysteine proteinase inhibitors p-chloromercuribenzoate and iodoacetamide. The activity of the enzyme is also unaffected by reaction with butanedione or phenylglyoxal in borate buffer, indicating the absence of essential arginyl residues.

The carboxyl group reagents Woodward's Reagent K and EDC both inactivate collagenase at pH 6.0. The time course of this inactivation is shown in Figure 1. Reaction with 10 mM Woodward's Reagent K reduces activity to 28% in 15 minutes, while 30 mM

Table I

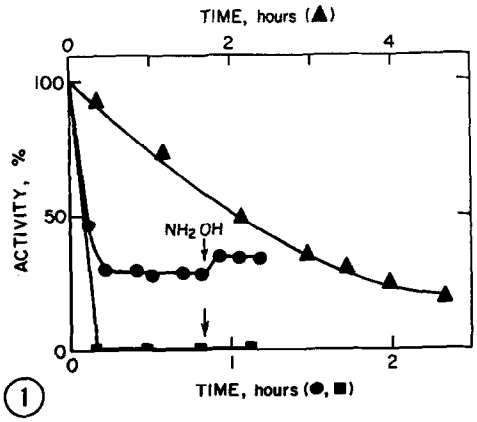
Effect of Various Reagents on the Activity of Clostridium histolyticum Collagenase

Reagent	Concentration, mM	Buffer[a]	Time, minutes	Relative[b] Activity
None				100
1,10-Phenanthroline	1	Tricine,pH 7.5	10	0
Ethylenediamine-tetraacetic acid	1	Tricine,pH 7.5	10	0
Diisopropylfluoro-phosphate	1	Tricine,pH 7.5	60	88
Phenylmethylsulfonyl fluoride	1	Tricine,pH 7.5	60	98
p-Chloromercuri-benzoate	1	Tricine,pH 7.5	60	96
Iodoacetamide	10	Tricine,pH 7.5	60	100
Butanedione	10	Borate, pH 8.0	60	96
Phenylglyoxal	10	Borate, pH 8.0	60	98
Tetranitromethane	3	Tris, pH 8.0	20	30
	8	Tris, pH 8.0	20	0
Acetic anhydride	2	Hepes, pH 7.5	10	39
Diketene	2	Hepes, pH 7.5	10	35
Trinitrobenzene sulfonate	5	Hepes, pH 7.5	60	10

[a] All buffers contain 10 mM $CaCl_2$.

[b] Percent activity after reaction, relative to the unmodified control.

reagent completely inactivates the enzyme in 10 minutes. At a concentration of 50 mM, EDC also inactivates the enzyme, but at a slower rate. Addition of hydroxylamine does not restore activity for either reaction. These results indicate that collagenase contains an essential carboxyl residue.



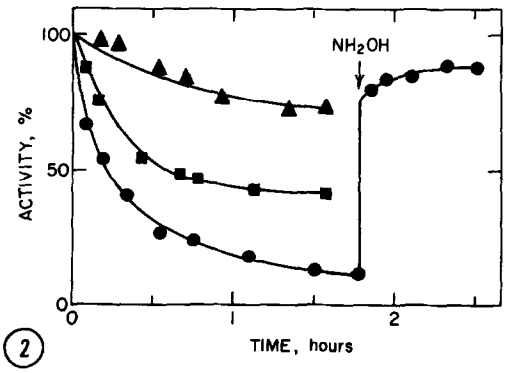


Figure 1: Changes in activity of Clostridium histolyticum collagenase on reaction with 50 mM EDC (▲, upper time scale), and with 10 mM (●) and 30 mM (■) Woodward's Reagent K (lower time scale) in 50 mM Mes, 10 mM $CaCl_2$, pH 6.0. The addition of hydroxylamine to a final concentration of 0.5 M is indicated by the arrow.

Figure 2: Changes in activity of Clostridium histolyticum collagenase on reaction with 5 mM acetylimidazole (■) and with 10 mM acetylimidazole in the presence (▲) and absence (●) of 10 mM Cbz-GPGGPA in 50 mM Hepes, 10 mM $CaCl_2$, pH 7.5. The addition of hydroxylamine to a final concentration of 0.5 M is indicated by the arrow.

Reaction with 5 and 10 mM acetylimidazole at pH 7.5 for 90 minutes lowers the activity of the enzyme to 41 and 12%, respectively, of the unmodified control (Figure 2). Almost full activity is restored by hydroxylamine. The presence of the substrate Cbz-GPGGPA (10 mM) partially protects the enzyme from inactivation, indicating that loss of activity is due to modification of a residue at the active site. The enzyme is also inactivated by tetranitromethane (Table I). Activity is reduced to 30 and 0 percent after reaction with 3 and 8 mM reagent, respectively, at pH 8.0 for 20 minutes. Collectively, these observations indicate the existence of an essential tyrosyl residue.

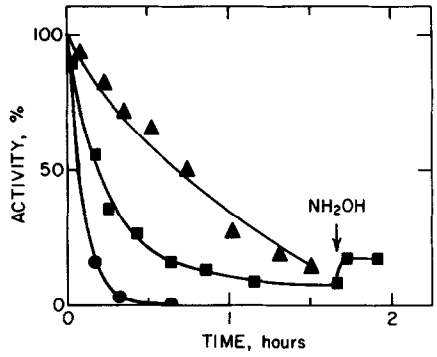


Figure 3: Changes in activity of Clostridium histolyticum collagenase on reaction with 30 mM diethylpyrocarbonate (●) and with 10 mM diethylpyrocarbonate in the presence (▲) and absence (■) of 10 mM Cbz-GPGGPA in 50 mM Hepes, 10 mM $CaCl_2$, pH 7.5. The addition of hydroxylamine to a final concentration of 0.5 M is indicated by the arrow.

Collagenase is completely inactivated by 30 mM diethylpyrocarbonate at pH 7.5 in 30 minutes (Figure 3). Activity is reduced to less than 10% on reaction with 10 mM reagent for 90 minutes. The presence of 10 mM Cbz-GPGGPA partially protects the enzyme from inactivation. Activity is not restored by addition of hydroxylamine, suggesting that ethoxyformylation of lysyl residues is responsible for the loss of activity. This conclusion is supported by the fact that the acylating reagents acetic anhydride and diketene also reduce the activity of the enzyme (Table I). Trinitrobenzene sulfonate also markedly lowers activity. Hydroxylamine fails to reactivate collagenase after any of these reactions.

Discussion: It has been recognized for some time that collagenase is a metalloenzyme (1). The enzyme is inhibited by 1,10-phenanthroline and this inhibition is reversed by zinc. It is also inhibited by ethylenediaminetetraacetic acid, which is reversed by calcium, but not zinc (6). Thus, the enzyme is thought to contain zinc at the active site and require calcium for stability,

much like thermolysin (7). These observations, as well as the insensitivity of the enzyme to seryl reagents such as diisopropylfluorophosphate and phenylmethylsulfonyl fluoride and cysteinyl reagents such as iodoacetamide and p-chloromercuribenzoate, are confirmed here (Table I). In addition, the chemical modification reactions carried out here indicate that the enzyme does not contain any essential arginyl residues, but does contain essential carboxyl, tyrosyl and lysyl residues.

The inactivation of collagenase by EDC and Woodward's Reagent K at pH 6.0 and the failure of hydroxylamine to reactivate the enzyme is characteristic of carboxyl group modification. Carboxypeptidase A is similarly inactivated by both types of reagents (8,9), and angiotensin converting enzyme is also inactivated by carbodiimides (10). The inactivation of collagenase by acetylimidazole, the reversal of this modification by hydroxylamine and the inactivation by tetranitromethane all indicate that the enzyme contains an essential tyrosyl residue (11,12). Since the enzyme is not sensitive to thiol reagents, cysteine can be ruled out as the site of these modifications.

Acylation of collagenase by acetic anhydride, diketene, and diethylpyrocarbonate markedly lowers activity and the reaction is not reversed by hydroxylamine. This indicates that modification of lysyl, rather than histidyl, cysteinyl or tyrosyl residues, is responsible for the loss of activity. Inactivation by trinitrobenzene sulfonate supports this conclusion. The existence of an essential lysyl residue is unusual, but has recently been reported for angiotensin converting enzyme, another neutral zinc metallopeptidase (10).

Only two previous reports of chemical modifications of collagenase have appeared. Solov'eva and Orekhovich (2) reported

that collagenase is inactivated by both acetylimidazole and succinic anhydride; the activity of the former reaction was restored upon addition of hydroxylamine, but the latter was not. These results are consistent with the modification of tyrosyl and lysyl residues, respectively, in agreement with results presented here. Takahashi and Seifter reported that photooxidation of histidyl residues inactivates collagenase (3). It is not possible to detect the presence of an essential histidyl residue in collagenase from the present data, since the reversible acylation of histidine would be masked by the irreversible acylation of lysine. Additional studies with reagents more specific for histidine are required to clarify whether this residue is at the active site of collagenase.

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