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Notes

The benzoylarginine peptidase from *Treponema denticola* (strain ASLM), a human oral spirochaete: evidence for active-site carboxyl groups

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Summary

The benzoylarginine peptidase of *Treponema denticola* (strain ASLM; a human oral spirochaete) was progressively and irreversibly inactivated by 1-(ethoxycarbonyl)-2-ethoxy-1, 2-dihydroquinoline, a carboxyl-group reagent. At acidic pH values, reaction of one mole of the modifier per active site of the enzyme resulted in total inactivation of the enzyme. Assuming that this modifier is a specific carboxyl reagent, the data suggest that the inactivation of the *T. denticola* benzoylarginine peptidase was caused by the modification of one carboxyl group located close to the active site of the enzyme. Results obtained with Woodward's reagent K (*N*-ethyl-5-phenylisoxazolium 3′-sulphonate) supported these findings. Carbethoxylation with diethylpyrocarbonate effectively inactivated the enzyme, and addition of hydroxylamine at pH 7.0 restored the activity almost totally, suggesting that the pyrocarbonate had reacted with tyrosyl or histidyl residues.

Introduction

Spirochaetes are helical, motile bacteria, many of which are found associated with a large number of eukaryotic hosts, from protozoa to mammals (Canale-Parola, 1977; Hardwood and Canale-Parola, 1984). One of the spirochaete genera, *Treponema*, is present in the mouth, intestinal tract, and genital areas of humans and other animals (Canale-Parola, 1977). The human oral spirochaetes have been associated with various types of periodontal disease (Listgarten, 1976; Listgarten and Hellden, 1978; Loesche *et al.*, 1982, 1985), the development of which may depend partly on the activity of proteolytic enzymes from these organisms. A previous paper (Ohta *et al.*, 1986) reported the purification and partial characterization of a benzoylarginine peptidase from *Treponema denticola* (strain ASLM) (implying that the enzyme was discovered in terms of its high activity on $N\alpha$-benzoyl-L-arginyl-*p*-nitroaniline (BAPNA) and $N\alpha$-benzoyl-L-arginyl-2-naphthylamine (BANA). This enzyme is a 50–65 kD heat-labile protein which has no activity on casein, haemoglobin, azocasein, azocoll, serum albumin and gelatin (Ohta *et al.*, 1986). It is possible that the enzyme plays a role in the nutrition of the spirochaetes.

Because *T. denticola* is pathogenic to man, it can be assumed that the proteolytic enzymes produced by this organism play an important role in the inflammatory reactions where the presence of *T. denticola* has been demonstrated. In spite of the previous advances (Canale-Parola, 1977; Hardwood and Canale-Parola, 1984) in the study of the physiology and morphology of these organisms, very little is known about their proteolytic enzymes. The purpose of this paper is to summarize our detailed experiments, which suggest the involvement of a carboxyl group in the activity of *T. denticola* benzoylarginine peptidase. The importance of this group was studied using 1-(ethoxycarbonyl)-2-ethoxy-1, 2-dihydroquinoline (EEDQ) as the principal modifying agent. This reagent has been found to modify essential carboxyl groups in enzymes and it has been claimed that it is highly specific for the activation and modification of such groups (Ho and Wang, 1980; Ting and Wang, 1980; Pougeois *et al.*, 1978; Saccomani *et al.*, 1981; Phelps and Hafeti, 1984; Mäkinen and Mäkinen, 1987).

Results and Discussion

Effect of EEDQ

Inactivation of the benzoylarginine peptidase by EEDQ at 40°C was determined in 0.05 M 2-(morpholine)-ethanesulphonic acid monohydrate (Mes) or 0.088 M phosphate buffers (at pH values ranging from 5.4–6.6, i.e. with a range

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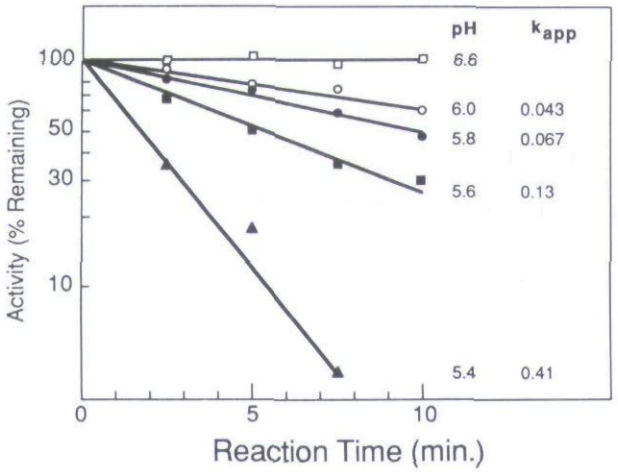

Fig. 1. Dependence of the rate of the EEDQ-caused inactivation of the *T. denticola* benzoylarginine peptidase on pH. The modifications were carried out in 88 mM phosphate buffers at 40°C. The pH values of the modification mixtures and the values of the apparent rate constants of inactivation (min^{-1}) are indicated.

of pHs at which the enzyme was sufficiently stable at 40°C). Figure 1 shows that the inactivation of the enzyme was clearly pH-dependent: the rate of inactivation increased with decreasing pH (compared with stability controls included at each pH values). EEDQ had no effect at pH 6.6., whereas at pH 5.4 the enzyme was totally inactivated in 7–10 min. Figure 2A shows that at 40°C, 0.45 mM EEDQ in 0.088 M phosphate buffer (pH 5.8), caused an almost 90% inactivation in 7.5 min; 2.0 mM reagent inactivated the enzyme totally in 2–3 min (not shown). The secondary plot of $\log(1/t_{0.5})$ versus log [EEDQ] produced a straight line with a slope of 0.99, suggesting that the observed rate of inactivation was dependent on [EEDQ] to the first power (Fig. 2B). This kinetic plotting method has been used to calculate the number of inhibitor molecules able to react per active site of an enzyme (Pougeois *et al.*, 1978; Phelps and Hafeti, 1984). Thus these results suggest that EEDQ has reacted with a reactive residue that is essential to the activity of the enzyme and that reaction of one mole of EEDQ per active site of benzoylarginine peptidase had probably caused this total enzyme inactivation. Collectively, these data showed that the inactivation of the enzyme by EEDQ was time-, concentration-, and pH-dependent. In previous studies (Pougeois *et al.*, 1978; Saccomani *et al.*, 1981; Phelps and Hafeti, 1984; Mäkinen and Mäkinen, 1987; Mäkinen *et al.*, 1982) such kinetic behaviour has been interpreted in terms of EEDQ reacting with an active carboxyl residue. The presence of BAPNA or BANA protected against the EEDQ-induced inactivation by 50–85% (both substrates were used at 5–25 mM concentrations).

Effect of Woodward's reagent K (WRK)

The effect of WRK on the activity of the enzyme was time- and concentration-dependent. The routine experiments were carried out in 0.1 M phosphate buffer, pH 5.8. Under these conditions, WRK (initial concentration 4.9 mM) destroyed more than 80% of the enzyme activity in 60 min (22°C) (not shown), while 1.5 mM WRK caused a 10% inactivation in the same time. The above substrates (5 mM) protected inactivation by WRK by 75%. The effect of WRK was also studied in different acidic pH values in 0.2 M Mes as described earlier (Mäkinen and Mäkinen, 1987; Mäkinen *et al.*, 1982). The inactivation rate increased with increasing pH of the modification medium, suggesting that WRK reacted with the deprotonated form of an enzyme carboxyl group. It has been claimed that WRK is a specific carboxyl-group reagent when tested under the conditions described above (Mäkinen and Mäkinen, 1987; Mäkinen *et al.* 1982).

Effect of 1-ethyl-3-(3-dimethyl-aminopropyl) carbodiimide hydrochloride (EDC)

The inactivation of the enzyme by EDC was studied in the presence of a nucleophile (glycine methyl ester) and

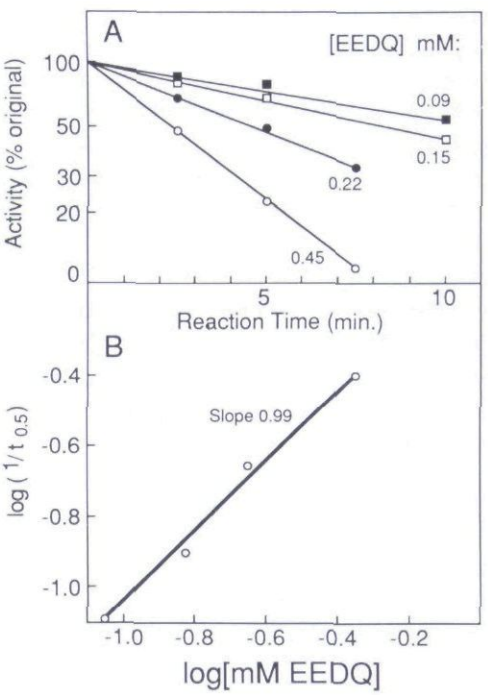

Fig. 2. Modification of the *T. denticola* benzoylarginine peptidase with EEDQ.
Panel A. Time course of inactivation of the enzyme. The enzyme (about 0.5 μM) was treated with EEDQ in 88 mM phosphate buffer, pH 5.8. The EEDQ concentrations present in the modification mixture are shown. Panel B. Secondary plot of $\log(1/t_{0.5})$ versus log[EEDQ]. The slope of the resulting curve is indicated. All modifications were performed at 40°C.

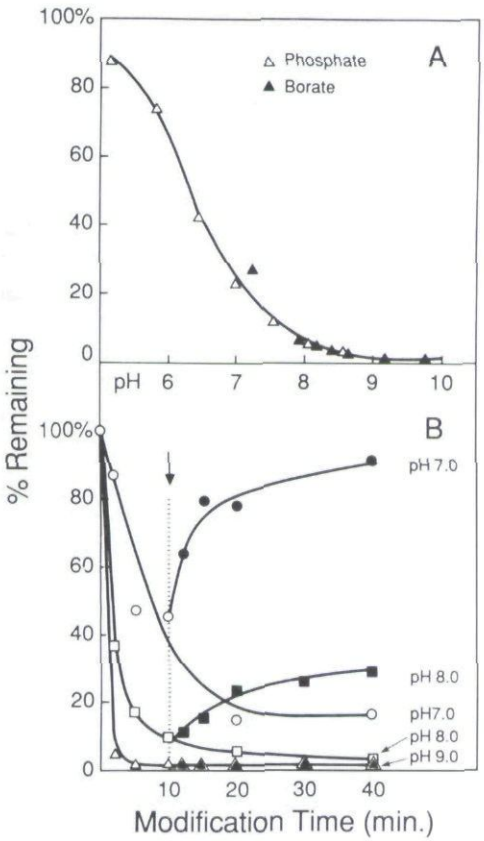

Fig. 3. Modification of the *T. denticola* benzoylarginine peptidase with DEP.
Panel A. Dependence of inactivation on pH, tested in 50 mM phosphate buffers or 50 mM borate buffers.
Panel B. Inactivation of the enzyme with DEP (open symbols) and reactivation of the inactivated enzyme with 0.1 M hydroxylamine (solid symbols). The inactivations were carried out either in 50 mM phosphate buffers (pH 7.0 and 8.0) or in 50 mM borate buffer (pH 9.0). The arrow at 10 min shows the addition of hydroxylamine.

without added nucleophile under conditions described elsewhere (Mäkinen *et al.*, 1982). 10 mM EDC inactivated the enzyme in a time-, concentration-, and pH-dependent manner, but the inactivation proceeded at a rate approximately one tenth of the rate observed with EEDQ. The presence of the nucleophile was necessary for this inactivation, and 5–25 mM substrates protected the enzyme against inactivation, as shown for EEDQ. Although EDC is less specific than EEDQ and WRK, the pattern of enzyme inactivation favours the possibility that this reagent may have reacted with a reactive carboxyl group. An aminopeptidase from *Aeromonas proteolytica* (Mäkinen *et al.* 1982) was shown to have an active carboxyl group and the inactivation of this enzyme by EDC was very similar to that of the benzoylarginine peptidase of *T. denticola*.

Effect of other reagents

The enzyme was treated at 22°C for up to 50 min with 0.1–5.0 mM concentrations of diethylpyrocarbonate (Mäkinen *et al.*, 1982; Mäkinen and Mäkinen, 1987; Miles, 1977). These modifier levels (the molar ratio of modifier to enzyme was 100:1) caused a fast time- and concentration-dependent inactivation of the enzyme (Fig. 3). The secondary plots of percent activity remaining versus modification time indicated the involvement of two or more consecutive (or simultaneous) carbethoxylation processes. Virtually no inactivation was observed at pH 5.0, whereas the process continued rapidly to an almost complete loss of enzyme activity at pH 8.5–9.0. The activity of the inactivated enzyme could be almost totally restored at pH 7.0 by the addition of 0.1 M hydroxylamine. It is possible that these results indicate the involvement of tyrosyl or histidyl residues in enzyme activity. Tetranitromethane, tested as described previously (Mäkinen *et al.*, 1982; Mäkinen and Mäkinen, 1987) caused a rapid inactivation of the enzyme. None of the diketones or ketone aldehydes (2,3-butanedione, 1,2-cyclohexanedione, phenylglyoxal, glyoxal and 2,3-pentanedione) inactivated the enzyme under non-photo-oxidative (Mäkinen *et al.*, 1982) conditions, suggesting that arginine residues may not be important for enzyme activity. Previous studies (Ohta *et al.*, 1986) showed that the activity of the benzoylarginine peptidase did not depend on SH-groups. The previous results (Ohta *et al.*, 1986), suggesting that the enzyme is not inhibited by metal chelators but is inactivated by diisopropylfluorophosphate, were verified.

Conclusions

The fact that the *T. denticola* benzoylarginine peptidase was progressively and irreversibly inactivated by selective carboxyl group reagents, EEDQ and Woodward's reagent K, should substantiate the presence of a reactive carboxyl group in this enzyme. With both modifiers the pH of the modification medium had a remarkable effect on the inactivation rate, which was more pronounced at acidic pH values. This was to be expected since a protonated carboxyl group is necessary for the reactivity of EEDQ (Pougeosis *et al.*, 1978; Phelps and Hafeti, 1984). The behaviour of the acidic portion of the rate versus pH curve (Ohta *et al.*, 1986) of the hydrolysis of BAPNA is in agreement with a reactive carboxyl group residue being present at or near the active site.

Although the inactivation of the enzyme upon treatment with EEDQ, WRK and EDC indicated the presence of carboxylate groups at the catalytic site, there is always the possibility of a side reaction or a conformational change indirectly altering the active site, or the accessibility of the reagent to the active site. Interpretation of protein-modification studies in general should consider this possibility;

such studies should ideally be accompanied by the use of different labelling techniques and immunologic structural probes (Ludwig *et al.*, 1985). EEDQ may cause the formation of inter- and intramolecular amide bonds (Saccomani *et al.*, 1981), leading to intramolecular cross-linking (Phelps and Hatefi, 1984; Ting and Wang, 1980). Also, treatment of an enzyme with diimides may result in cross-linking of the enzyme protein (Phelps and Hatefi, 1984; Pougeois *et al.*, 1978). Therefore the carboxyl reagents employed in this study may have triggered such long-range conformational changes. It is improbable, however, that three unrelated labelling reactions (using EEDQ, WRK, and EDC) could have induced the same extent of long-range changes. Furthermore, the protein amino groups are not reactive at those low pH values at which the inactivation of the enzyme by EEDQ was most rapid. This lessens the occurrence of EEDQ-induced cross-linking between those groups. Consequently, considering all the supportive information presented in this study, it is very likely that EEDQ and WRK (and possibly EDC) had reacted with a carboxyl group located near the active site, or at the active site, of the *T. denticola* benzoylarginine peptidase.

The present evidence suggests that diethylpyrocarbonate may have reacted with either histidyl or tyrosyl residues which are important to enzyme activity. The inactivation was most rapid at alkaline pH values, indicating that lysyl, tyrosyl or histidyl residues may have reacted with the modifier. However, the reaction was reversed at neutral pH values by hydroxylamine, which removes the carbethoxy group from modified hystidyl or tyrosyl residues but does not remove that group from modified lysyl or cysteine residues (Melchior and Fahrney, 1970). Therefore, cysteine and lysine may be ruled out as essential amino acid residues reacting with diethylpyrocarbonate.

There has been no comprehensive chemical study of any *Treponema* peptidase. The spirochaetes have been difficult to cultivate and this fact has been particularly true for oral treponemes, which display strict nutritional requirements. The present communication represents the first effort to elucidate the nature of the active amino acid residues of a spirochaete enzyme. Provided that EEDQ and Woodward's reagent K can be regarded as specific carboxyl-group reagents, it appears that the activity of the *T. denticola* benzoylarginine peptidase depends on the reactivity of at least one such acidic group. In the classification of Neurath (Neurath *et al.*, 1967; Neurath, 1984, 1985) peptidases that are sensitive to diisopropylfluorophosphate and whose activities depend on an active carboxyl group are designated as serine proteases I (EC 3.4.21): this includes trypsin, with which the *T. denticola* enzyme shares some substrate-specificity characteristics (Ohta *et al.*, 1986).

Experimental procedures

EEDQ, EDC, and Woodward's reagent K were purchased from Serva. Other reagents were obtained from Sigma. The *T. denticola* benzoylarginine peptidase was purified as previously described (Ohta *et al.*, 1986).

Enzyme activity was determined as previously described (Ohta *et al.*, 1986) at 30 °C in assay mixtures consisting of 0.3 ml of 0.1 M Tris-HCl (pH 7.8), 0.1 ml of 1.0 mM BAPNA (or BANA) solution, 0.1 ml of water and 0.1 ml of enzyme (diluted with buffer). Protein was determined according to the Bradford method (Whitaker and Granum, 1980).

Chemical modification of the enzyme with EEDQ and EDC was carried out in mixtures consisting of 1.0 ml of 0.05 M Mes (normally pH 5.5) or 0.088 M phosphate buffers (normally pH 5.8), and of 10 μg of enzyme. After 5 min standing at 30°C (EDC and WRK) or at 40°C (EEDQ), various amounts of methanolic EEDQ (the final methanol concentration in stock solution was 10–50%), or aqueous EDC were added to the modification mixture. The modifier solutions were freshly prepared. The extent of modification was followed at 30°C (EDC, WRK), or at 40°C (EEDQ) during periods of 30–120 min with small (10–20 μl) aliquots withdrawn from the mixtures at the proper time intervals. Appropriate methanol and pH controls were included. Otherwise, the recommendations of the literature (Ho and Wang, 1980; Ting and Wang, 1980; Pougeois *et al.*, 1978) were followed. The treatment of the enzyme with EEDQ was carried out at 40°C to ensure complete dissolution of the reagent at higher concentrations. In protection experiments, the protectors were added to buffered enzyme 5 min before the addition of the modifier. Inactivation of the enzyme with WRK was carried out as previously described (Saccomani *et al.*, 1981; Mäkinen and Mäkinen, 1987). The experiments with diethylpyrocarbonate (DEC) were performed as recommended by Miles (1977).

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