PHOTOCHEMICAL INACTIVATION OF *AEROMONAS* AMINOPEPTIDASE

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1. Introduction

Aeromonas aminopeptidase (AAP) which has been discovered and studied by Prescott and his coworkers [1,2], was recently shown to undergo irreversible photochemical inactivation in the presence of 2,3-butanedione (BD) [3]. This reaction is of interest since BD and related diketones and ketone aldehydes have been widely used as specific probes for the active arginyl residues of enzymes. The previous [3] and the present studies on the BD-sensitized photochemical inactivation of AAP revealed, however, that this process is accompanied by extensive structural changes in the enzyme protein, including destruction of possibly all enzyme tryptophyl residues. The purpose of this communication was to provide additional information about the BD-sensitized photochemical inactivation of AAP. It was shown that the destruction of peptidase and esterase activities occurred simultaneously. The results were also consistent with an all-or-none loss of activity hypothesis (i.e. no partially inactivated enzyme active sites exist). Progressive enzyme inactivation was accompanied by a progressive destruction of the enzyme. It is predictable that these phenomena are common among proteins and peptides containing tryptophan which may be a primary target of the triplet stage sensitizers (^{3}BD in the present case). These results also warn us from drawing conclusions from enzyme modification data obtained with BD and related compounds, unless the photochemical and photophysical aspects are carefully considered.

2. Materials and methods

AAP was purified in principle as in [2]. AAP activity was determined with L-leucyl-*p*-nitroanilide (Leu-*p*NA) or L-phenylalanine methyl ester (PME) substrates in 10 mM tricine buffer (pH 8.0) containing 0.1 mM $ZnSO_4 \cdot 7\ H_2O$ and 0.2 M KCl. The photochemical modification of AAP in the presence of BD was carried out in the visible light as in [3]. Irradiation intensities of 0.1–1700 microEinsteins (μE; 1 μE = 10 lux) were used and the intensities were measured with a phototube [3]. The kinetic constants were determined and molecular permeation chromatography and disc electrophoresis carried out as in [3,4]. The chemicals and their sources were given in [3].

3. Results

Fig.1 demonstrates the absolute requirement of the BD-sensitized inactivation of AAP for light; in the dark (light intensity ~0.1 μE), the enzyme retained full activity even in the presence of 5×10^5 molar excess of BD and 0.05 M borate. Upon exposure to visible light (100 μE) the inactivation of AAP proceeded rapidly. The esterase and peptidase activities of AAP were affected by the inactivation process in a similar manner. The rates of inactivation were clearly light-dependent; irradiation at 1 μE for 10 min hardly inactivated AAP, whereas at 20 μE the enzyme lost 80% of its activity. BD was, however, a poor inhibitor of the hydrolysis of Leu-*p*NA. 1-Phenyl-1,2-propanedione (PPD) caused a similar photochemical inactivation of AAP as BD.

Plots of log AAP esterase activity as a function of the BD-sensitized photochemical inactivation time showed the inactivation to approximate first order kinetics with respect to time (fig.2). Fig.2 also shows that the plot of log $(1/t_{0.5})$ *vs* log [BD] produced a straight line with a slope of 0.83 which equals to the number of molecules of BD reacting with each active site of AAP to produce an inactive EI complex. The BD-sensitized photochemical inactivation of AAP thus

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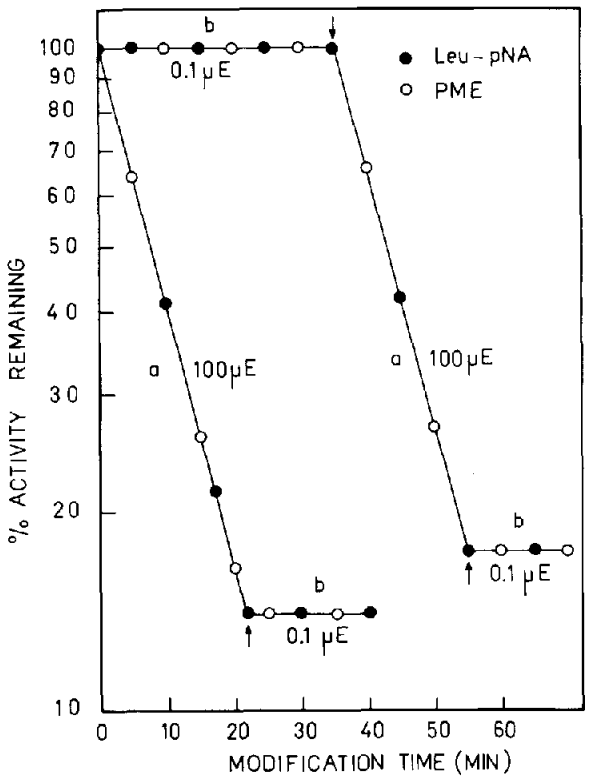

Fig.1. BD-sensitized photochemical inactivation of AAP (0.25 μM) in 50 mM borate buffer (pH 7.2) in visible light. Two identical reaction mixtures were initially treated either in visible light (a) or protected from light (b). The arrows indicate either the beginning or ending of the irradiation period. [BD] = 1 mM.

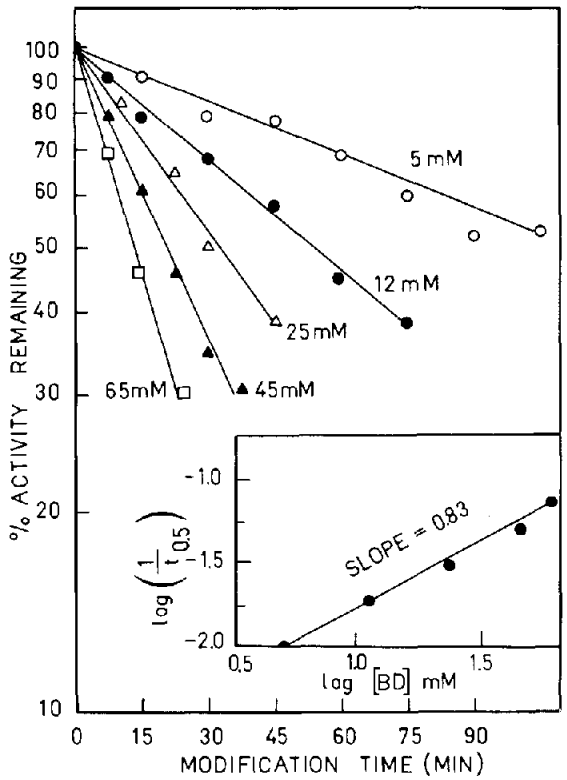

Fig.2. Effect of the concentration of BD. Loss of activity of AAP in the presence of BD in 50 mM borate buffer (pH 7.2) in visible light (25 μE). Insert: A plot of log $(1/t_{0.5})$ *vs* log [BD], where $t_{0.5}$ is the time required for 50% inactivation. Tested with PME.

initially resulted from the reaction of 1 essential amino acid residue/active site of AAP with 1 molecule of BD. This result was also obtained when studying the peptidase activity of AAP [3]. The subsequent, secondary reactions of AAP in the presence of light and BD were, however, more complicated, as shown below.

Fig.3 demonstrates that the treatment of AAP with BD in light (100 μE) resulted in a loss of tryptophan fluorescence. This experiment thus confirmed extensive destruction of the tryptophyl residues of AAP. These residues were not destroyed by treatment of AAP with BD in the dark (0.1 μE). L-Thiol-2-histidine (TH) was the most effective protector of AAP against the BD-sensitized photochemical inactivation. Quenching was observed at very low protector levels; even at molar ratios of 2:1 (quencher to AAP), protection was observed for a considerable time. TH protected the peptidase and esterase activities in a similar way. NaN_3 (10 mM) and TH (0.1 mM) which are known quenchers of photooxidation [3,5,6] halted, in addition to the BD-sensitized photochemical inactivation of APP, the destruction of tryptophyl residues as well. These reactions proceeded rapidly when O_2 was introduced into the reaction mixture. Molecular permeation chromatography on Sephadex G-50 superfine and disc electrophoresis on polyacrylamide gel also showed that progressive enzyme inactivation was accompanied by a progressive destruction of the enzyme molecule.

Measurements on 100% photoinactivated AAP demonstrated that both activity and fluorescence losses were irreversible, the former permitting a further characterization of the enzyme inactivation process. For this purpose, a Lineweaver-Burk analysis was per-

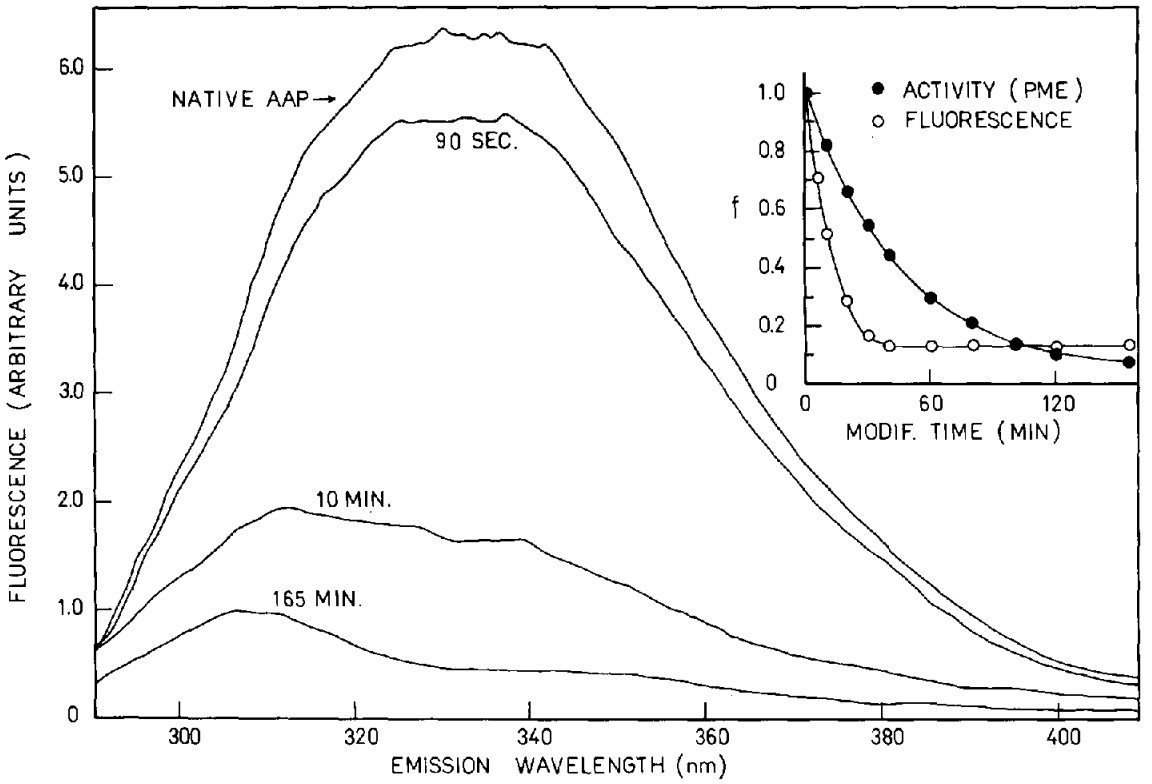

Fig.3. Loss of tryptophan fluorescence during the BD-sensitized photochemical inactivation of AAP. The emission spectra upon excitation at 280 nm of both the native AAP and inactivated AAP were determined. AAP (1.65 mg) was modified in 25 mM borate buffer (pH 7.2) in visible light (20 μE) with 0.1 mM BD [3]. Insert: Fractional loss of either fluorescence or AAP activity (with PME) as a function of time.

formed both on control (i.e., BD-treatment in the dark; BD thereafter removed by dialysis [3]) and irradiated, BD-treated samples. Table 1 shows that for samples that had lost 17, 47, 54 and 68% of their activity, the ratio of V_{max} (irradiated) to V_{max} (control) ranged from 0.83–0.15, whereas the ratio of K_m (irradiated) to K_m (control) was ~1.0. These results are consistent with an all-or-none loss of activity hypothesis, i.e., no partially inactivated enzyme active sites exist. The results were similar with both peptidase and esterase substrates.

Table 1
Effect of BD-sensitized photochemical inactivation on V_{max} and K_m

Enzyme activity remaining (%)	V_{max} (irrad.) / V_{max} (control)	K_m (irrad.) / K_m (control)
83	0.83	0.98
53	0.36	0.96
46	0.21	1.02
32	0.15	0.96 (mean 0.98)

The enzyme was inactivated in the presence of BD (1 mM) and visible light (100 μE) to the activity levels indicated [3]. As a control, native AAP was used

4. Discussion

The results showed that the BD-sensitized photochemical inactivation is a photooxidation process which destroys both the peptidase and esterase activity of the enzyme. The photochemistry of BD is relatively well known [7–11], but the photochemical aspects have been largely ignored by enzyme and protein chemists who have exploited BD as a specific reagent of the active arginyl residues of enzymes. These results

thus suggest that most of the previous arginine modification studies with BD and related α-diketones should be repeated by considering the photochemical and photophysical aspects involved. Moreover, the BD-modified enzymes should be subjected to both amino acid analysis and spectral studies before drawing conclusions from the involvement of active arginyl residues. It is possible that all previous modification studies with BD, unless performed in the dark, have involved tryptophan losses as well. These losses may have been overlooked and explained simply as destruction of tryptophan during amino acid analysis only.

The studies carried out in this laboratory indicate that TH is a novel and by far a more effective singlet oxygen quencher and free radical scavenger than N_3^- which has widely been used for these purposes. TH, in possessing a dual quenching activity due to its SH and imidazole groups, was shown in this laboratory to protect even against the UV light-induced photochemical destruction of acetylcholine esterase.

The BD-sensitized photochemical inactivation of AAP should not be regarded as a special case. On the contrary, it may be expected that a great number of proteins and peptides will undergo a similar inactivation process and that in addition to BD, PPD and 2,3-pentanedione [3], several other dicarbonyls (ketones and ketone aldehydes) will cause similar effects, either in the visible or UV region. These views are supported by recent, independent studies [3,12, 13].

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