Oxidation, photosensitized by certain diketones, of enzymes and protection against such oxidation by histidine derivatives

K. K. MÄKINEN* and P.-L. MÄKINEN†

*Department of Biochemistry, Institute of Dentistry, University of Turku, SF-20520 Turku 52, Finland; and †Department of Forensic Medicine, University of Turku, SF-20500 Turku 50, Finland

(Received 12 February 1982)

Bovine milk lactoperoxidase, eel acetylcholinesterase, and Aeromonas aminopeptidase were photooxidized and inactivated in broad-spectrum visible light in the presence of 2,3-butanedione and 1-phenyl-1,2-propanedione. Methylglyoxal caused similar effects at 254 nm. 2-Thiol-L-histidine and 3-methyl-L-histidine protected the enzymes against photoinactivation more effectively than N3−, even at a molar ratio of 2:1 (protector to enzyme). These compounds also delayed the photoinactivation of acetylcholinesterase, induced by ultraviolet light.

2,3-Butanedione and related carbonyl compounds have been widely exploited in the probing of active arginyl residues of enzymes. The suitability of these compounds for this purpose has been questioned, however, on the basis of previous photochemical studies (Mäkinen & Mäkinen, 1982b). It was recently shown that 2,3-butanedione, methylglyoxal, and 2,3-pentanedione act as sensitizers in the photochemical inactivation of enzymes (Fliss & Viswanatha, 1979; Gripon & Hofmann, 1981; Mäkinen, 1981; Mäkinen et al., 1982; Mäkinen & Mäkinen, 1982a,b). Our preliminary studies later showed that this dicarbonyl-sensitized photooxidation of enzymes can be effectively prevented by small amounts of 2-thiol-L-histidine and 3-methyl-L-histidine. The ability of these compounds to maintain full enzyme activity under strong photooxidative conditions suggests that this quenching phenomenon may have important practical applications.

Many imidazole derivatives are themselves subject to photooxidation and thus it may not be surprising that they should exert a protective effect against enzyme inactivation. In spite of this fact, we were unable to find articles dealing with the protective effects of the present imidazole derivatives. The ability of these compounds to protect the u.v.-light-induced inactivation of acetylcholinesterase was considered totally new. Moreover, we found that these imidazole derivatives were by far better protectors than N3−, which has frequently been exploited as a quencher. Furthermore, the imidazole derivatives do not generally inhibit metalloenzymes as azide does. Although the photochemistry of imidazole derivatives is well known, their enzyme-protecting effect has received less attention. This

©1982 The Biochemical Society
property has only rarely been exploited in biochemistry and practical applications. The purpose of this communication is to report the dicarbonyl-sensitized photoinactivation of bovine milk lactoperoxidase, electric-eel acetylcholinesterase, and *Aeromonas* aminopeptidase, and the ability of histidine derivatives to protect the enzymes against this inactivation.

**Materials and Methods**

The chemicals and their sources were previously mentioned (Mäkinen et al., 1982; Mäkinen & Mäkinen, 1982a). Electric-eel acetylcholinesterase (Type V) was obtained from Sigma (St. Louis, MO, USA), and bovine milk lactoperoxidase from P-L Biochemicals (Milwaukee, WI, USA), and *Aeromonas* aminopeptidase was purified as previously described (Prescott & Wilkes, 1976). The imidazole derivatives were purchased from Koch-Light (Colnbrook, Buckinghamshire, England).

The photochemical oxidation of enzymes with dicarbonyls in broad-spectrum visible light was carried out as previously (Mäkinen et al., 1982; Mäkinen & Mäkinen, 1982a), but using a wider range of light intensity [0.1 microeinstein (µE) to 1700 µE; 1 µE = 10 lux]. The enzymes were irradiated in quartz cells at 0°C in 50 mM borate buffer, pH 7.2, or in 10 mM phosphate buffer, pH 7.0 (Mäkinen et al., 1982; Mäkinen & Mäkinen, 1982a). The light intensities were measured with a phototube. The photochemical destruction of acetylcholinesterase in u.v. light (292 nm) was performed as previously described (Bishop et al., 1980) using a Hitachi Fluorescence Spectrophotometer MPF-2A. The rate of photochemical inactivation and the spectral changes of the enzymes were studied as a function of time after adding the protector (quencher, final concentration 0.1 µM-1.0 mM) and the sensitizer (final concentration 1.0 mM-100 mM) in the reaction mixtures. Photooxidation of the enzymes was carried out by including the following reaction mixtures and treatments: (1) E, irr.; (2) E, dark; (3) E+S*, irr.; (4) E+S*, dark; (5) E+P+S*, irr.; (6) E+P, irr.; (7) E+P, dark, where irr. = irradiation, E = enzyme, P = protector, and S* = sensitizer.

The u.v. spectra were recorded with a Perkin-Elmer Double Beam Spectrophotometer 124. Acetylcholinesterase activity was assayed with *N*-acetylthiocholine iodide as substrate (Ellman et al., 1961). The activity of lactoperoxidase was determined with guaiacol (Chance & Maehly, 1964). The assay of aminopeptidase has been previously described (Prescott & Wilkes, 1976).

**Results**

**Photooxidation of enzymes**

The dicarbonyl compounds 2,3-butanedione and 1-phenyl-1,2-propanedione caused a rapid and irreversible photochemical inactivation of all three enzymes (shown for 2,3-butanedione in Fig. 1 D-F). The inactivation process was accompanied by extensive structural changes in the enzyme molecules, as indicated by the virtually total loss of tryptophan fluorescence (Fig. 1 A-C) and disappearance of the
Fig. 1. Protection by 2-thiol-L-histidine (TH) and \( \text{N}_3^- \) against the 2,3-butanedione (BD)-sensitized destruction of tryptophyl residues (panels A–C) and activity loss (panels D–F) of three enzymes. The enzymes (0.1–0.4 \( \mu \text{M} \)) were incubated with 1 \( \text{mM} \) BD under illumination of 25 \( \mu \text{E} \) (broad-spectrum visible light), and samples were withdrawn and dialyzed in the dark (Mäkinen et al., 1982). The ordinates of panels A–C indicate arbitrary units of tryptophyl content as determined from fluorescence measurements on the dialyzed samples at 330 nm (excitation at 292 nm). The controls, indicated in the 'Materials and Methods' section, produced fluorescence curves which overlapped those given by the native enzymes. ACE = acetylcholinesterase; LPO = lactoperoxidase; AAP = Aeromonas aminopeptidase.
characteristic protein absorption curve in the u.v. region (shown for 2,3-butanedione in Fig. 2). In the u.v. and visible region, both 2,3-pentanedione and 1-phenyl-1,2-propanedione caused qualitatively similar effects as 2,3-butanedione. Methylglyoxal inactivated the enzymes at 254 nm but not in the visible region.

The absolute requirement of the dicarbonyl-sensitized inactivation of enzymes for light was demonstrated; in the dark (light intensity < 0.1 μE), the enzymes retained full activity even in the presence of 5 x 10^5 molar excess of 2,3-butanedione in 0.05 M borate. Upon
exposure to light the enzyme inactivation and the structural changes indicated proceeded rapidly. Plots of log enzyme activity as a function of dicarbonyl-sensitized photochemical inactivation time showed the inactivations to approximate first-order kinetics with respect to time. In broad-spectrum visible light, 1-phenyl-1,2-propanedione caused slightly faster enzyme inactivation than the other diketones tested. Since 2,3-butanedione is, however, by far more frequently used in enzyme modification studies for probing active arginy1 residues, the results are shown for this compound only.

Protection against photooxidation by histidine derivatives

When small amounts of 2-thiol-L-histidine or 3-methyl-L-histidine were previously added to the enzyme modification mixture, no activity and fluorescence loss occurred (Fig. 1). These compounds were by far more effective protectors than NaN₃ and a large number of other, potential singlet oxygen quenchers and free radical scavengers (Mäkinen et al., 1982; Mäkinen & Mäkinen, 1982a). The efficacy of protection was evidenced by the fact that detectable protection of the aminopeptidase was observed during a period of 10 min at a molar ratio of 2:1 (protector to enzyme), i.e. quencher concentrations as low as 0.5-1.0 µM were effective, although the modifier-to-enzyme ratio was as high as 4 x 10⁵ (molar ratio). For a shorter period of time (<1 min), protection was detected at equimolar concentrations. The imidazole derivatives used were more effective protectors than histidine, imidazole, methionine, cysteine, tyrosine, and all histidyl dipeptides studied.

Although the effects of both imidazole derivatives were qualitatively similar, the best quenchers exerted a certain type of selectivity. 3-Methyl-L-histidine, for example, protected lactoperoxidase more effectively than 2-thiol-L-histidine did. At a molar ratio of 200:1 (2-thiol-L-histidine to lactoperoxidase), the thiol derivative and 1-methyl-L-histidine produced an approximate protection of 30%, while 3-methyl-L-histidine, at the above molar ratio, displayed virtually 100% protection. On the other hand, the thiol and 3-methyl derivatives protected the aminopeptidase strongly and to an equal extent, while the 1-methyl derivative appeared to be less effective.

Protection against u.v.-light-induced inactivation of acetylcholinesterase

Acetylcholinesterase undergoes a severe destruction process in u.v. light (Bishop et al., 1980). Relatively small concentrations of the thiol and 3-methyl derivatives of histidine retarded the u.v.-light-induced inactivation and the loss of tryptophan fluorescence of acetylcholinesterase which proceeded in the absence of any exogenous sensitizer (Fig. 3). N₅⁺ and other compounds listed above were by far poorer protectors than 2-thiol-L-histidine and 3-methyl-L-histidine. For example, while 0.6 mM 2-thiol-L-histidine protected the enzyme by 82% against the u.v.-light-induced inactivation, compounds like phenol, 1-methyl-L-histidine, methanol, L-cysteine, 2-mercaptoethanol, L-histidine, and imidazole, all at 0.6 mM, caused 5-20% protection only when the irradiation time was 20 min.
Fig. 3. Protection by 2-thiol-L-histidine (TH) and N\textsubscript{3}\textsuperscript{-} (both at 60 \textmu{}M) against the u.v.-light-induced loss of tryptophyl residues and activity loss of acetylcholinesterase in the absence of added sensitizers. 13.8 \textmu{}g of enzyme was treated in 0.1 M phosphate buffer, pH 8.0, in a quartz cell at 292 nm (20 nm band width) as described elsewhere (Bishop et al., 1980). Other details were as for Fig. 1.

Discussion

Previous literature has shown that 2,3-butanedione, upon absorption of photons, generates free radicals (Bentrude & Darnall, 1968; Pitts & Wan, 1966). These and numerous other photochemical and photophysical studies with 2,3-butanedione have not been considered in enzyme modification studies aimed at elucidating the role of arginyl residues of enzymes and binding proteins. Consequently, it was recently shown that 2,3-butanedione sensitizes photochemical inactivation and extensive structural changes in enzyme molecules, thus indicating that this carbonyl compound cannot be regarded as a specific arginine probe unless the reaction mixture is protected from light (for a review, see Mäkinen & Mäkinen, 1982b). The present study suggested that 1-phenyl-1,2-propanedione also causes similar photochemical reactions and that several types of enzymes are photooxidizable under the conditions indicated.

N\textsubscript{3}\textsuperscript{-}, methanol, and related nucleophilic species have been commonly used to intercept photooxidation reactions (Hasty et al., 1972; Nilsson et al., 1972) and N\textsubscript{3}\textsuperscript{-} has been shown to act as a singlet oxygen quencher (Matheson et al., 1975). Our experiments showed, however,
that the histidine derivatives used produced a more significant decrease in the lifetime of the excited molecules than N$_3^-$ or any of the other compounds tested, including several unsubstituted amino acids. Higher quencher levels produced complete protection for several hours under strong photooxidative conditions. The efficacy of the quenchers used suggested that they may also bind to the active site of the enzymes, thereby exerting more effective protection than other simple molecules. In accordance with photochemical literature, it may be assumed that the histidine derivatives acted as a quencher of singlet oxygen or the excited states of the sensitizers, or as free radical scavengers. The protective effect could also be ascribed to competition between the imidazole derivatives and the enzyme molecules for singlet oxygen (and/or free radicals). These compounds can anyway be considered better protectors against the photochemical inactivation of enzymes than N$_3^-$, which frequently inhibits enzymes, especially those containing a metal cofactor. Lactoperoxidase, for example, was strongly inhibited by N$_3^-$. These results demonstrated that enzymes are readily photooxidizable in the presence of dicarbonyl reagents that have previously been used as arginyl probes. This process is effectively prevented by certain histidine derivatives. These compounds can also prevent u.v.-light-induced enzyme inactivation.

References

Hasty N, Merkel PB, Radlick P & Kearns DR (1972) Tetrahedron
Lett. 49-51.
Mäkinen KK, Mäkinen P-L, Wilkes SH, Bayliss ME & Prescott JM
Matheson IBC, Etheridge RD, Kratowich NR & Lee J (1975) Photo-
chem. Photobiol. 21, 165-171.
Nilsson R, Merkel PB & Kearns DR (1972) Photochem. Photobiol. 16,
117-124.
Pitts JN Jr & Wan JKS (1966) in The Chemistry of the Carbonyl