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Photodynamic cross-linking of proteins
IV. Nature of the His–His bond(s) formed in the rose bengal-photosensitized cross-linking of *N*-benzoyl-L-histidine

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Abstract

The photodynamic (photosensitized) cross-linking of *N*-benzoyl-L-histidine (Bz-His) as a model system was examined as part of a continuing study of the role of His–His intermolecular cross-links in the photosensitized cross-linking of proteins. The illumination of Bz-His in the presence of rose bengal (RB) bound to water insoluble plastic beads in 0.1 M sodium phosphate buffer of pH 7.4 resulted in the covalent cross-linking of the His derivative. The main dimeric cross-linked product (**1**) was isolated using a preparative silica gel 60 column and purified by preparative reverse phase HPLC. The chemical structure of the cross-link was determined using MS, 2D NMR spectral methods and other standard techniques. Product **1** was found to be a dimer of two His residues between the δ2-carbon of one residue (photo-oxidized to the carbonyl functionality at the ε1-carbon) and the ε2-nitrogen of the other residue. The formation of His–His cross-links was mediated by singlet oxygen, as would be expected with RB as the sensitizer. A mechanism for the formation of the cross-link was proposed in which the first step was the 1,4-cycloaddition of singlet oxygen to the Bz-His imidazole ring to give an unstable endoperoxide. This then underwent changes followed by nucleophilic addition and the elimination of one molecule of water to give **1**.
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Keywords: Singlet oxygen pathway; Cross-linking pathways/mechanism

Abbreviations: ACN, acetonitrile; Bz-His, *N*-benzoyl-L-histidine; DTT, dithiothreitol; HPMA, *N*-(2-hydroxypropyl)methacrylamide; MB, methylene blue; PDT, photodynamic therapy; PP, protoporphyrin; RB, rose bengal; SDS, sodium dodecyl sulfate; TFA, trifluoroacetic acid

1. Introduction

The photosensitized cross-linking of proteins is involved in several kinds of processes of biomedical importance, including the photodynamic therapy (PDT) of tumors and other diseases [1,2], the laser welding of tissues [3] and the preparation of tissue-derived biomaterials for implants [4]. Also, some of the pathological effects produced by sunlight, including the photoaging of skin [5] and the induction of cataracts, appear to involve cross-link formation in proteins [6,7]. Previous studies have shown that reactions involving His residues play a key role in the photodynamic cross-linking of proteins as mediated by the singlet oxygen pathway [8–10]. Model studies using ^{14}C labeled free amino acids and amino acids attached to sepharose gel have demonstrated that His can cross-link with itself, Lys, Cys, Trp, Tyr, and even Arg (much lower efficiency) on photosensitized treatment [11,12]. Recently, we unequivocally demonstrated the photosensitized formation of intermolecular His–His and His–Lys cross-links using HPMA co-polymers containing side chains terminating in His or Lys [9]. The key role played by His in the photodynamic cross-linking of proteins was also confirmed by studies with a model protein, ribonuclease A [10].

It is difficult to identify modified amino acid residues in photodynamically-treated proteins and to determine their locations. It is also difficult and laborious to isolate the cross-linked amino acid moieties. Consequently, low molecular weight His and imidazole derivatives have been used to study the detailed reaction mechanisms. Unfortunately, these photo-oxidation studies were performed either in an organic solvent [13] or in an alkaline aqueous solution (pH = 11) [14]. These nonphysiological reaction

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conditions may lead to different mechanisms and kinetics of photo-oxidation; this could result in the formation of intermediate and final products different from those occurring in biological systems. In addition, previous studies have shown that photodynamically-produced His–His cross-links are unstable under alkaline conditions [14,15]. Despite the large amount of research done previously, the detailed mechanisms of cross-link formation involving His are still unknown.

This paper describes the use of *N*-benzoyl-L-histidine (Bz-His) as a model compound for elucidating the chemical nature and mechanisms of the formation of His–His cross-links as produced by photosensitized reactions under somewhat physiological conditions (0.10 M aqueous buffer, pH 7.4). Protection of the α-amino group of His with the benzoyl group prevents the participation of the amino group in cross-linking reactions. In addition, the UV-absorbing benzoyl group permits the spectroscopic detection of Bz-His and its photo-oxidized and cross-linked products. Rose bengal (RB), which sensitizes primarily by the singlet oxygen mechanism in aqueous solution [16], was used as the photosensitizer; the RB was covalently coupled to polystyrene beads, which facilitated its removal from the reaction system. The main reaction product (**1**) was isolated and purified. Its chemical structure was determined by MS, 2-D NMR spectroscopy and other analytical methods. A reaction mechanism for the photosensitized formation of the His–His dimer was proposed.

2. Experimental details

2.1. Materials and general methods

The Bz-His, from BACHEM Bioscience, was used as received. RB beads (Sensitox^{TM} II) were obtained from Hydron Laboratories. The other chemicals were of the highest purity commercially available. All of the reactions were carried out in double distilled water. Light spectral absorption measurements were made with a Perkin–Elmer Lambda 9 spectrophotometer. TLC was performed using an aluminum backed silica gel 250 μM layer from Whatman (Kent, England) and HPLC was performed using a Beckman pumping system equipped with either a preparative or an analytical C18 VYDAC^{TM} column. NMR experiments were performed on a Varian Unity 500 instrument.

2.2. Photosensitized cross-linking of Bz-His

The photocross-linking reaction systems contained 65 mg RB beads and 300 mg of Bz-His in 60 ml of 0.1 M Na phosphate buffer (pH 7.4). The oxygen concentration was 0.22 mM (air-saturated), as measured with an oxygen electrode. The reaction solution was illuminated in a double-walled reaction vessel kept at 25°C with circulating water. A 500 W incandescent lamp with a heat-absorbing filter and a Corion LL-400 long-wave length pass filter to remove UV radiation was used. The reaction vessel was placed 20 cm away from the light source. The incident light energy flux was ~3 W cm^{-2}. Illumination lasted for 8 h.

2.3. Isolation and purification of the photocross-linked product(s) of Bz-His

After illumination, the RB beads were filtered off, and the solution was immediately frozen and lyophilized. The resulting solid was dissolved in absolute ethanol (EtOH), and the insoluble buffer salt was removed by filtration. The EtOH solution was condensed by rotoevaporation at room temperature to give an oily residue. The residue (~0.9 g from three batches) was dissolved in 12 ml of EtOH : H_2O (8 : 2), applied to a pre-equilibrated silica gel 60 column (55 cm × 4.5 cm), and eluted with the same solvent at a flow rate of 1.1 ml min^{-1}. The cross-linked product(s) was separated from the starting Bz-His and lyophilized. Fractions were pooled and purified by preparative reverse phase HPLC using a C18 VYDAC^{TM} column (22 mm × 250 mm, a linear gradient from 0.1% TFA/88% H_2O/12% acetonitrile (ACN) to 0.1% TFA/21.6% H_2O/78.4% ACN over 95 min at a flow rate of 2 ml min^{-1}). The main product fraction was collected and lyophilized. This purification procedure was repeated. The majority of the starting material remained unreacted. The chemical structures of the photo-oxidized product(s) of Bz-His (other than the cross-linked material) have not been determined. The conversion of Bz-His to its dimer(s) was about 4.7%. The main dimer (20 mg), which accounted for more than 85% of all the dimerized products, was obtained as a amorphous white solid. TLC (silica gel, EtOH : H_2O (7 : 3)), one gray spot under UV lamp, $R_f = 0.69$; $[\alpha]_D^{25} = -17°$ ($c = 0.445$, H_2O); UV (H_2O) $\lambda_{max} = 217$ nm, $\varepsilon = 2.14 \times 10^4$; IR (film) ν_{max} 3690–2150 br (COOH), 1690 (C=O), 1662 (C=O), 1579 (C–C) cm^{-1}; ^{1}H NMR (DMSO-d_6, 500 MHz), see Table 1; ^{13}C NMR (DMSO-d_6, 500 MHZ), see Table 1; ESMS (50 : 50 H_2O : CH_3OH with 1% CH_3COOH) obs. $(M+H)^+$ *m/z* (relative intensity) 533 (100); LRFABMS(CH_3OH/NBA) obs. $(M+H)^+$ *m/z* 533 and $(M+Na)^+$ 555; HRFABMS (MeOH/NBA/PEG) obs. $(M+H)^+$ *m/z* 533.1795 (calc. for $C_{26}H_{25}N_6O_7$, 533.1785). The hydrolysis of the cross-linked product was monitored by imidazole ring assay using Pauly's reaction. Bz-His reacts with Pauly's reagent to form a reddish colored product, while the cross-linked product does not [17].

3. Results

3.1. Isolation and characterization of the photocross-linked product of Bz-His

The use of RB, methylene blue (MB), or protoporphrin (PP) as photosensitizers appeared to produce the same photo-oxidation products as determined by TLC. This would

Table 1
NMR assignments for the Bz-His cross-linked product[a] (DMSO-d_6)

Atom No.	^{13}C δ	^{1}H δ	HMBC (8 Hz)
1	172.4		
2	51.2	4.65 (m, 1H)	C1, C3, C4, C10
3	24.8	2.84 (m, 2H)	C1, C2, C4, C5
4	112.8		
5	112.7		
6 (NH)		10.37 (s, 1H)	C4, C5, C7
7	151.6		
8 (NH)		10.37 (s, 1H)	C4, C5, C7
9 (NH)		8.64 (d, 1H, J = 8.0)	C1, C2, C10
10	166.2		
11	133.7		
12	127.3	7.76 (d, 1H, J = 7.5)	C10, C14, C16
13	128.3	7.45 (t, 1H, J = 7.5)	C10, C11, C15
14	131.5	7.52 (t, 1H, J = 7.5)	C11, C12, C16
15	128.3	7.45 (t, 1H, J = 7.5)	C10, C11, C13
16	127.3	7.76 (d, 1H, J = 7.5)	C10, C12, C14
1′	172.3		
2′	51.8	4.65 (m, 1H)	C1′, C3′, C4′, C10′
3′	27.1	3.14 (m, 2H)	C1′, C2′, C4′, C5′
4′	133.3		
5′	119.7	7.36 (s, 1H)	C4′, C7′
6′ (N)			
7′	136.9	8.70 (s, 1H)	
8′ (N)			
9′ (NH)		8.79 (d, 1H, J = 8.0)	C2′, C10′
10′	166.4		
11′	133.7		
12′	127.4	7.82 (d, 1H, J = 7.5)	C10′, C14′, C16′
13′	128.3	7.44 (t, 1H, J = 7.5)	C10′, C11′, C15′
14′	131.5	7.51 (t, 1H, J = 7.5)	C11′, C12′, C16′
15′	128.3	7.44 (t, 1H, J = 7.5)	C10′, C11′, C13′
16′	127.4	7.82 (d, 1H, J = 7.5)	C10′, C12′, C14′

[a] All the spectra were acquired in DMSO-d_6 at 500 MHz. J values were reported in Hertz. Assignments were aided by HMQC, GCOSY, DEPT, GHMBC and reference NMR spectra of Bz-His.

Table 2
NMR assignments for the Bz-His cross-linked product[a] (DMSO-d_6 + D_2O)

Atom No.	^{13}C δ	^{1}H δ	HMBC (8 Hz)
1	172.6		
2	51.7	4.60 (m, 1H)	C1, C3, C4, C10
3	25.0	2.87 (m, 2H)	C1, C2, C4, C5
4	114.2		
5	112.7		
6 (NH)			
7	152.0		
8 (NH)			
9 (NH)			
10	167.3		
11	133.6		
12	127.7	7.71 (d, 1H, J = 7.5)	C10, C14, C16
13	129.0	7.46 (t, 1H, J = 7.5)	C11, C12, C15
14	132.3	7.52 (t, 1H, J = 7.5)	C11, C12, C16
15	129.0	7.46 (t, 1H, J = 7.5)	C11, C13, C16
16	127.7	7.71 (d, 1H, J = 7.5)	C10, C12, C14
1′	172.5		
2′	52.0	4.60 (m, 1H)	C1′, C3′, C4′, C10′
3′	26.8	3.15 (m, 2H)	C1′, C2′, C4′, C5′
4′	132.6		
5′	120.9	7.39 (s, 1H)	C5, C7′
6′ (N)			
7′	137.1	8.92 (s, 1H)	C5, C4′, C5′
8′ (N)			
9′ (NH)			
10′	167.6		
11′	133.8		
12′	127.8	7.76 (d, 1H, J = 7.5)	C10′, C14′, C16′
13′	129.0	7.45 (t, 1H, J = 7.5)	C11′, C12′, C15′
14′	132.4	7.51 (t, 1H, J = 7.5)	C11′, C12′, C16′
15′	129.0	7.45 (t, 1H, J = 7.5)	C11′, C13′, C16′
16′	127.8	7.76 (d, 1H, J = 7.5)	C10′, C12′, C14′

[a] All spectra were recorded in DMSO-d_6 with one drop of D_2O at 500 MHz. J values were reported in Hertz. Assignments were supported by HMQC, GCOSY, DEPT, and GHMBC.

be expected since all of the above sensitizers primarily produce singlet oxygen.

The main cross-linked product (**1**) was isolated using a preparative silica gel 60 column [EtOH : H_2O (7 : 3)], and purified on a reverse phase HPLC column. This compound was found to have the molecular formula $C_{26}H_{24}N_6O_7$, with 18 units of unsaturation, as determined from the ESMS, LR-FABMS, HRFABMS and NMR spectroscopy data (Tables 1 and 2). The UV spectrum of **1** was similar to that of Bz-His. It was not racemized, and gave no reaction with Pauly's reagent. In addition, acid (6N HCl) catalyzed hydrolysis (20 h, 105°C) of **1** liberated 1 mol equivalent of His (the Bz group was also hydrolyzed). Base catalyzed hydrolysis (pH 8.0–11.5) slowly produced Bz-His; the rate of hydrolysis increased with increasing pH as measured by Pauly's reaction (data not shown). ESMS analysis of **1** in [D_2O : MeOH-d_4 (50 : 50), 1% TFA-d] by ESMS showed a peak at m/z 540 (MW = 538), indicating that **1** contained six exchangeable protons.

The IR spectra of Bz-His and **1** were very similar. The absorption peaks at 1690 and 1662 cm^{-1}, and the ^{13}C chemical shifts of δ 151.6, 172.4, 172.3, 166.2 and 166.4 were assigned to the carbonyl components of the ureido group (NHCONH) (C7), carboxylic acid (C1/C1′) and amide bond (C10/C10′) (peak obscured) functionalities, respectively.

Assignments of most of the protons and carbons (H2, H3, H9, H12–16, C1–3, C10–16) were facilitated by comparison with the NMR spectra of the starting material Bz-His, and confirmed by analysis of the HMQC, COSY, DEPT and HMBC experiments (Tables 1 and 2). Carbon resonances at δ 133.5 and δ 119.7 were assigned to C4′ and C5′, respectively, using correlations observed from the protons at δ 4.65 (H2′) and δ 3.14 (H3′) in HMBC experiments. The assignment of C4 (δ 112.8) and C5 (δ 112.7) was difficult because they shared similar chemical shifts. However, addition of one drop of D_2O to a solution of **1** in DMSO-d_6 altered the chemical shifts of C4 and C5 to δ 114.2 and δ 112.7, respectively, and facilitated their assignments using long-range correlations from the protons H2 and H3 in HMBC experiments. The chemical shifts of C4 and C5 were observed to resonate upfield of C4′ and C5′ because, after dimerization, C4 and C5 are no longer aromatic carbons. The carbon resonating at δ 151.6 was assigned to the ureido carbon C7, based on its distinctive chemical shift and HMBC correla-

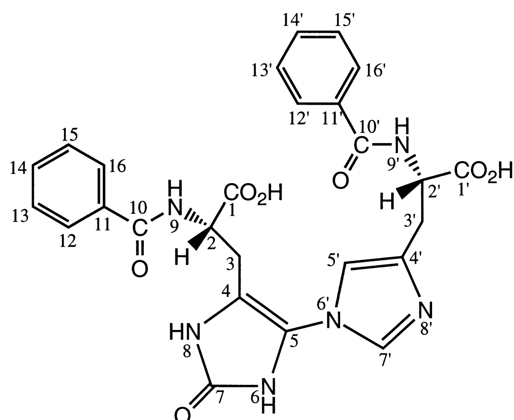

Fig. 1. The chemical structure of the cross-linked dimer of Bz-His (compound **1**) produced by illumination in the presence of RB, as determined by NMR experiments. Compound **1** was a dimer of two His residues between the δ2-carbon of one and the ε2-nitrogen of the other.

tions observed from the protons H6 and H8. The carbon resonance at δ 136.9, which was similar to the chemical shift of the same carbon on the imidazole ring of Bz-His, was assigned as C7′ using a correlation from the proton at δ 7.36 (H5′) in an HMBC experiment. The proton signal at δ 8.70 (H7′) appeared as a broad peak in a ^{1}H NMR spectrum measured in DMSO-d_6. However, this peak shifted downfield and sharpened when one drop of D_2O was added, enabling the correlations between H5′ and C7′, and H7′ and C5′ to be observed in an HMBC experiment. In addition, a weak coupling between H5′ and H7′ was observed in a COSY experiment for both Bz-His and **1**. The long-range correlations observed from the protons H5′ (δ 7.39) and H7′(δ 8.92) to C5 (δ 112.7) in an HMBC experiment were measured in DMSO-d_6 and a drop of D_2O allowed the assignment of the point of dimerization as being between C5 and N6′. The structure of **1** was established (Fig. 1).

4. Discussion

The imidazole group in Bz-His is the only available functional group in cross-linking since the α-amino group is protected and the carboxylic group usually does not participate in photocross-linking reactions with RB, MB, and PP as sensitizers [11]. In proteins, except for those at the C and N terminals, amino and carboxylic groups of His form amide bonds with other amino acid residues in the polypeptide chain. Our previous studies have shown that, under the reaction conditions used, protecting the amino and/or carboxylic group(s) of His had no effect on the quantum yield of oxygen uptake during RB-sensitized photo-oxidation [9]. Thus, Bz-His should serve as a suitable model compound to study the detailed chemical nature of the His–His cross-link(s) involved in the photocross-linking of proteins in biological situations.

Model studies using free and gel-bound ^{14}C labeled amino acids demonstrated that intermolecular His–His cross-link(s) were formed on illumination in the presence of singlet oxygen-producing photosensitizers such as RB, MB and PP [11,12]. The His–His cross-link(s) could possibly form between a modified and an unmodified imidazole residue, between two modified residues, or between a modified residue with the α-amino group of His. Recently, we demonstrated cross-link formation between two imidazole groups of His by using HPMA co-polymers containing side chains terminating in His residues with RB as the sensitizer [9].

The sensitized photo-oxidation of His is typically mediated by the singlet oxygen pathway [18]. Several research groups have proposed that the imidazole of His is photo-oxidized through the formation of endoperoxides [13,14]. Actually, unstable endoperoxide intermediates have been shown to be formed by the 1,4 cycloaddition of singlet oxygen to the unprotonated imidazole ring, as detected at very low temperatures [19]. Reaction of singlet oxygen with Bz-His may produce two endoperoxide isomers since the imidazole has two isomers, the τ and π forms. The endoperoxides are extremely unstable and produce two imidazolones by cleavage of the O–O bond and the prototropy of the two endoperoxides [13,14]. Each intermediate contains two electrophilic loci, C4 and C5, which could be attacked by nucleophiles from the same or another Bz-His molecule to form dimers.

In the present study, one possible detailed reaction mechanism for the formation of **1** (**1b**) is proposed, as shown in Fig. 2. Theoretically, four possible types of dimers can form. Dimers **1a** and **2a** are further able to eliminate one H_2O molecule and form a carbon carbon double bond. They contain a stable conjugated resonance structure. Obviously, **1** experiences the least steric hindrance during the nucleophilic addition process and has the most stable conjugated resonance structure.

Another possibility is that the elimination of a water molecule could occur to give hydroxyimidazolinones (after heterolytic fragmentation of the unstable endoperoxides) before the nucleophilic addition of another Bz-His molecule. In this case, the two types of hydroxyimidazolinones in the scheme are dehydrated, yielding the same intermediate. Nucleophilic addition of another Bz-His molecule followed by tautomerization will end up with the formation of compound **1**.

The photo-oxidation of Bz-His at pH 11.0, as sensitized by RB, produces a complex mixture of more than 17 products with six dimers (yields ranging from 1 to 14%). The dimers were isolated and characterized by techniques such as ^{1}H NMR, MS, IR and UV spectroscopy, and their chemical structures proposed [14]. One of the dimers (yield 3%) had the same chemical structure as **1**, before the elimination of one water molecule. This suggests that strong alkaline conditions (pH = 11) are unfavorable for the dehydration of the compound since all of the six dimers isolated were not dehydrated [14]. Depending on the pH, and other reaction conditions, dimers prior to and after the elimination of H_2O

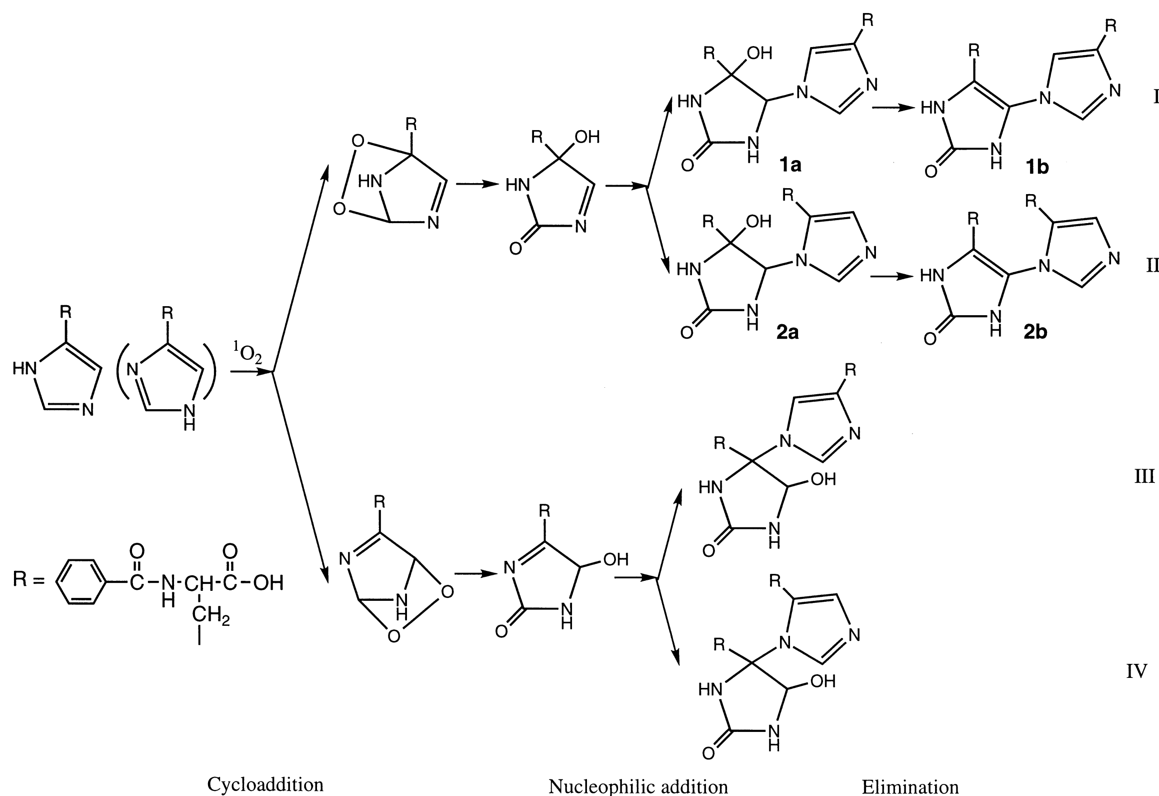

Fig. 2. Possible cross-linking pathways for the formation of dimerized products of Bz-His as a result of sensitized photo-oxidation with RB.

may be generated, and the yields of each dimer may be different. In addition, the dimers formed may differ in stability.

The dimers formed were observed to release 1 mol equivalent of Bz-His on alkaline hydrolysis [14]. We also observed that the acid hydrolysis (6N HCl) of **1** yields 1 mol equivalent of free His (from Bz-His), and unknown product(s) by HPLC using the ninhydrin method for detection. No aspartic acid was identified from hydrolyzed **1** by amino acid analysis. In addition, **1** was unstable under alkaline conditions and hydrolyzed slowly. Our previous studies also show the instability of RB photogenerated His–His cross-link(s) in His-containing HPMA co-polymers and RNase A under alkaline conditions (data not shown); the cross-links were also ruptured by treatment with boiling water for 5 min in the presence of sodium dodecyl sulfate/dithiothreitol (SDS/DTT) [10]. The degradation of **1** might be expected to occur between N6 and C7, N8 and C7, or C5 and N6′, with the production of further oxidized product(s).

N-benzoylaspartic acid (yield 15%), *N*-benzoylaspartylurea (4%) and *N*-benzoylasparagine (8%) etc. have been reported to be isolated as photo-oxidization products of Bz-His [14]. Another group reported that aspartic acid (2%) and urea were the final photo-oxidized products of His [20]. Aspartic acid and other unidentified products were also obtained after 6N HCl hydrolysis of *N*-benzoylhistidine methyl ester that had been illuminated in a dilute methanolic solution with MB as the sensitizer [13]. The different results from these researchers may result from varied reaction conditions. In our study, we did not attempt to isolate photo-oxidized products other than the dimers since we were investigating the nature of the cross-link(s) formed.

In biological situations, only **1** is expected to be produced since the steric hindrance in protein macromolecules is greater than that of the Bz group. Also, the formation of less stable dimers would be unlikely in proteins. In addition, the intermediates produced by photo-oxidation could interact with other residues in proteins such as Lys, Trp, Tyr, Cys, and Arg, and yield other cross-linked products having different stabilities [11,12]. The intermediates, potential dark reactions, and products in biological systems could be even more complicated because of the presence of other biomolecules such as DNA, RNA, lipids and amino acids. Photodynamic treatment can produce cross-links between proteins and these types of molecules [2,17,21].

Our next step is to study His-mediated photocross-linking as modeled by HPMA co-polymers containing enzymatically cleavable optimized oligopeptide side chains terminating in His, and to compare the His–His cross-linked products with those observed in the present study. Our ultimate goal is to elucidate the nature of His–His cross-link(s) formation in model proteins, and the influence of His modification on the activity of certain biologically active proteins such as enzymes. Since His residues in proteins probably play the most important role in the photodynamic cross-linking of proteins involved in PDT, cataract formation, skin photoaging and other processes, understanding the detailed mecha-

nism of His cross-link formation could help develop methods for the prevention of these pathological processes, and optimize the efficiency of photodrugs used for PDT while minimizing possible side effects.

Acknowledgements

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