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SHORT COMMUNICATION

Rapid Hydrolysis and Slow α,β-Dicarbonyl Cleavage of an Agent Proposed to Cleave Glucose-Derived Protein Cross-Links

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ABSTRACT. The putative protein glycation cross-link cleaving agent *N*-phenacylthiazolium bromide (PTB) underwent hydrolysis and cyclic hemithioacetal formation under physiological conditions to form two isomeric 2,3-dihydro-4-formyl-2-hydroxy-2-phenyl-1,4-thiazines: at pH 7.4 and 37°, the rate constant $k_{Hydrolysis}$ was $(2.6 \pm 0.1) \times 10^{-4}$ sec and the chemical half-life was *ca.* 44 min. The α,β-dicarbonyl cleavage reaction only competed effectively with the hydrolysis when the α,β-dicarbonyl substrate was at nonphysiological high levels. The high concentrations of PTB (10–30 mM) used previously to demonstrate chemical and biochemical activity also lead to acidification of incubation media. The mechanism of action of PTB now requires reappraisal. BIOCHEM PHARMACOL **57**;3:303–307, 1999. © 1998 Elsevier Science Inc.

KEY WORDS. glycation; advanced glycation end products; thiazolium; α,β-dicarbonyl; nonsulphydryl cross-link; aminoguanidine

The non enzymatic glycosylation of proteins by glucose and formation of end-stage adducts (AGE)† has been implicated in pathological mechanisms associated with diabetic complications, macrovascular disease, chronic renal insufficiency, Alzheimer's disease, and aging. Non sulphydryl covalent cross-linking of proteins has been found in thickening of blood capillary basement membranes in microvascular and macrovascular disease and protein aggregation in amyloidosis and cataract [1–4]. Some protein cross-links have been characterized: bis(lysyl)imidazolium derivatives [5] and pentosidine [6]; cross-links formed from 1,4-dideoxy-1-alkylamino-2,3-hexosulose have also been proposed [7]. These modifications are irreversible in physiological systems being decreased only by protein degradation and renewal. It was therefore of interest that the thiazolium derivative PTB [8] cleaved α,β-dicarbonyl compounds, and this may be a pharmacological strategy to remove α,β-dicarbonyl cross-links. PTB does not cleave imidazolium cross-links but it may cleave their α-oxoaldehyde precursors, glyoxal, methylglyoxal and 3-deoxyglucosone. Investigating this, we found rapid hydrolysis of PTB, acidification of incubation media, and reduction of disulphide bonds by PTB hydrolysis products under physiological conditions that indicated previous studies with PTB require reinterpretation.

MATERIALS AND METHODS

Materials

PTB was prepared as described [8]. The yield was 74%. Reversed phase HPLC analysis confirmed high purity of the preparation (see below). HSA (globulin-free, lyophilized powder) was purchased from Sigma Chemical Co. Ltd. and 1-phenyl-1,2-propanedione was purchased from Aldrich Chemical Co. Ltd. HSA highly modified by glucose-derived AGE (AGE-HSA) and [^{14}C]aminoguanidine (1 mCi/mmol) were prepared as described [9, 10].

Studies of PTB Hydrolysis and α,β-Dicarbonyl Cleavage

PTB (10 mM) was incubated in 50% methanol containing 50 mM sodium phosphate buffer (pH 7.4) under nitrogen at 37°. Aliquots of the reaction mixture (5 μL) were withdrawn at 50-min intervals for 400 min and analysed by HPLC. The column was a 10 cm × 0.8 cm NOVAPAK™ octadecyl silica cartridge with 1-cm precolumn in a 10 × 8 radial compression module. The mobile phase was 50 mM of sodium phosphate, pH 7.4, with a linear gradient of 25–50% methanol from 0–30 min and isocratic 50% methanol from 30–40 min. The eluate absorbance was monitored at 249 nm. The hydrolysis products formed were then synthesised on a preparative scale. PTB (10 mM, 28.4 mg) was incubated in 50% methanol containing 50 mM sodium phosphate buffer, pH 7.4 and 37°, for 6 hr. The product mixture was lyophilized to dryness, redissolved in 5

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† *Abbreviations:* AGE, advanced glycation end products; HSA, human serum albumin; and AGE-HSA, HSA highly modified by glucose-derived AGE; and PTB, *N*-phenacylthiazolium bromide;.

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mL of 10% methanol containing 50 mM sodium phosphate buffer, pH 7.4. The components were separated by preparative HPLC (column, 2.5 × 10 cm octadecyl silica cartridge in a Waters 25 × 10 radial compression module; mobile phase, 10 mM sodium phosphate, pH 7.4, with 10% (v/v) methanol, 9 mL/min; detection, absorbance at 280 nm; sample volume, 3 × 1.7 mL). The eluate fraction of retention interval 22–38 min was collected and lyophilized to dryness to yield the hydrolysis products which were characterised by ^{1}H and ^{13}C NMR spectroscopy and FAB mass spectrometry. The hydrolysis of PTB was also investigated by absorbance spectrophotometry and by ^{1}H and ^{13}C NMR spectroscopy in 50 mM sodium phosphate buffer in 50% d_4-methanol in deuterium oxide, pH 7.4 and 37°. The cleavage of the model α,β-dicarbonyl, 1-phenyl-1,2-propanedione, was then reinvestigated under the conditions described [8], analysing the reaction mixture by reversed phase HPLC as above. Products were identified by chromatographic identity with authentic standards and FAB mass spectrometry of lyophilized peak retention fractions.

Estimation of α,β-Dicarbonyl Compounds in HSA and AGE-HSA

HSA and AGE-HSA (1 mg/mL, 1 mL) were incubated with [^{14}C]aminoguanidine (200 μM) in PBS for 1–4 h at 37°. The amount of [^{14}C]aminoguanidine bound irreversibly to HSA and AGE-HSA increased in the initial 2-hr period and remained at a constant maximum value thereafter. After incubation, the protein was washed 5 times with PBS (5 mL) by ultrafiltration on CF25 membrane cones (Amicon); the final filtrate had <1 cpm. Aliquots of the washed protein solution were counted and assayed for protein content by the Lowry method. The molar equivalents of α,β-dicarbonyl compounds in the proteins was deduced by calculating the amount (mmol) of [^{14}C]aminoguanidine bound per mol of protein. The total count of [^{14}C]aminoguanidine per experiment (200 nmol) was 16881 ± 1280 cpm (N = 3).

Effect of PTB on Thiol Content of HSA and AGE-HSA

HSA and AGE-HSA (1 mg/mL) were incubated in sodium phosphate buffer (50 mM, pH 7.4) with and without 3 mM of PTB at 37° for 400 min. A metal ion chelator was not added to these incubations to maintain identity with the incubations of Vasan *et al.* [8]. The samples were then stored at −196° until further processing. Aliquots (400 μL) were concentrated to 50 μL by ultrafiltration, using a membrane spin filter (12 kDa cut-off, Whatman; with centrifugation of 10,000 *g* for 12 min). Samples were then diluted with ice-cold PBS (400 μL) and concentrated again. This was repeated a total of 6 times, discarding the filtrate. Finally, the samples were reconstituted in 400 μL of ice-cold PBS, protein content assayed by the Lowry method, and thiol content determined by reaction to end point with Ellman's reagent [11]. Thiol content of HSA and AGE-HSA is given as molar equivalents of HSA derivative.

RESULTS AND DISCUSSION

When 10 mM of PTB was incubated in 50% methanol containing 50 mM of sodium phosphate buffer, pH 7.4, 37° and under nitrogen, analysis of the PTB solution gave evidence of the rapid degradation of PTB. Typical chromatographic analysis data are given in Fig. 1a, where the products formed were only partially resolved. A similar incubation was performed on a preparative scale and purification of the products enabled the characterisation of the hydrolysis products of PTB. PTB hydrolysed to form two isomeric 2,3-dihydro-4-formyl-2-hydroxy-2-phenyl-1,4-thiazines (Fig. 2). The ^{1}H NMR spectrum of thiazine hydrolysis products gave (δ_H ppm (J Hz) in d_6-DMSO: H-3A 3.36 ($J_{5A,5B} = -11.5$), H-3B 4.52 ($J_{5A,5B} = -11.5$), H-6 5.72 and 5.97 ($J_{2,3} = 7.8$), H-5 7.10 and 7.16 ($J_{2,3} = 7.8$), 4-*N*-formyl 7.98 and 8.51; 2-phenyl H-2,6(2H) 7.56, H-3,5(2H) 7.44, H-4 7.37 ($J_{o,m} = 6.2$, $J_{m,p} = 7.7$). Proton-coupled ^{13}C NMR gave: (δ_C ppm ($J_{C,H}$ Hz, multiplet structure): thiazine isomer C-3 49.5 ($J_{5,5} = 144$, t) and 54.9 ($J_{5,5} = 144$, t), C-2 77.8 (s) and 78.8 (s), C-6 100.6 ($J_{2,2} = 180$, d) and 104.8 ($J_{2,2} = 179$, d), C-5 117.4 ($J_{3,3} = 180$, d), and 121.6 ($J_{3,3} = 179$, d), 4,*N*-formyl 162.3 ($J_{4,N\text{-formyl},4,N\text{-formyl}} = 202$, d), and 163.2 ($J_{4,N\text{-formyl},4,N\text{-formyl}} = 206$, d); 2-phenyl C-1 142.8, C-2,6 129.8 ($J_o = 161$, d), C-3,5 127.4 ($J_m = 162$, d), C-4 124.9 ($J_p = 160$). FAB mass spectrometry of the hydrolysis products gave a molecular ion with $(m + 1)/z = 222$. The relative proportion of the two isomers was approximately 1:1. PTB hydrolysis was also studied under similar conditions in deuterated solvents where the thiazolium H-2 proton and the α-protons of the *N*-phenacyl group of PTB and the thiazine H-2 protons exchanged with solvent deuterium, as expected [12]. The absence of a ketone carbonyl in the ^{13}C NMR spectrum and in equivalence of the H-3 protons in the ^{1}H NMR spectrum was consistent with the cyclisation of the intermediate ene-thiols to form isomeric 2,3-dihydro-4-formyl-2-hydroxy-2-phenyl-1,4-thiazines [13], which accounts for their similar chromatographic retention times. They were formed by hydrolysis of the thiazolium group of PTB and intramolecular hemithioacetal formation. Thiazolium compounds typically undergo hydroxide-catalysed hydrolysis at physiological pH via a C-2 tetrahedral reaction intermediate [14]. Hydrolysis of 3-(2-oxoalkyl/aryl)thiazolium derivatives, of which PTB is an example, hydrolyse to form 2-alkyl/aryl-2,3-dihydro-4-formyl-2-hydroxy-1,4-thiazines, and two endo- and exo-rotamers of the formamide group are formed [13].

The rate of hydrolysis of PTB decreased markedly after 100 min. This was due to a progressive decrease in pH as the PTB hydrolysed, consuming one equivalent of hydroxide ions: the pH decreased from 7.4 to 6.7 over the 400-min incubation period studied. The reaction conditions used to

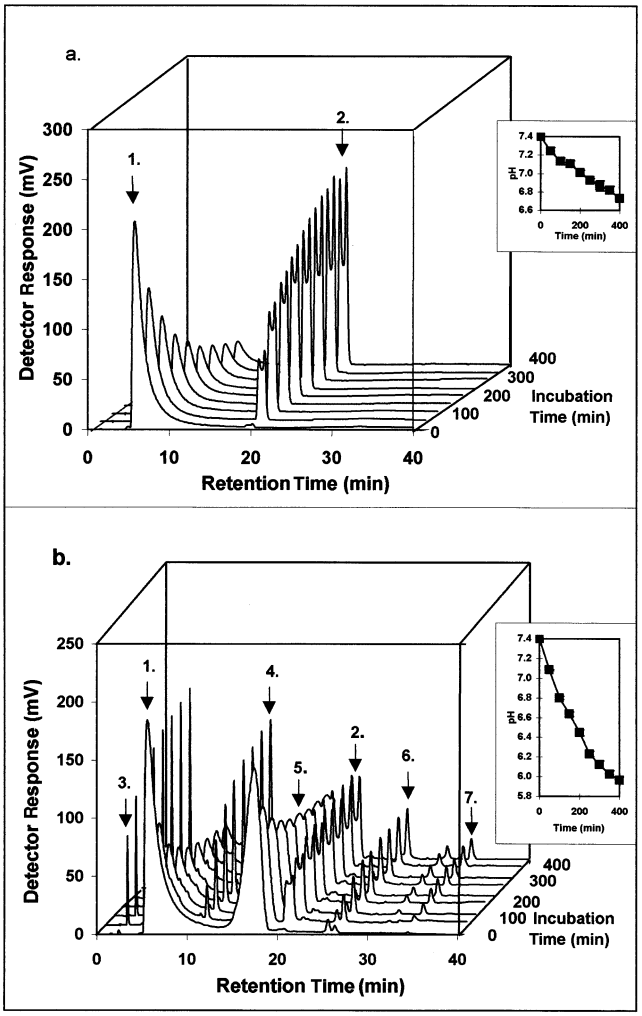

FIG. 1. Competing hydrolysis and α,β-dicarbonyl cleavage reactions of PTB. PTB (10 mM) was incubated in 50% methanol containing 50 mM of sodium phosphate buffer, pH 7.4 and 37°, under nitrogen in the absence (a) and presence (b) of 22 mM of 1-phenyl-1,2-propanedione. Figure insets show the pH of the reaction mixtures; data are means ± SD. (N = 3). Analyte peak maximum retention times: peak 1, PTB 5.6 min; peak 2, PTB hydrolysis products 20.0 and 20.7 min; peak 3, benzoic acid (cleavage product) 2.4 min; peak 4, 2-hydroxy-*N*-phenacyl-*N*-(2-thioethen-1-yl)propanamide ((m + 1)/*z* = 267; hydrolysed, acetyl-derived cleavage product) 11.2 min; peak 5, 1-phenyl-2-oxopropanone 17.4 min; peak 6, benzoin 26.4 min (cross-link reformation); peak 7, 2-(1-hydroxybenzyl)-*N*-phenacylthiazolium (cleavage product) 33.5 min. Other: unidentified trace contaminant in 1-phenyl-2-oxopropanone 25.7 min, unidentified product 30.8 min.

study the hydrolysis of PTB were identical to those employed in the previous study of AGE cleavage by PTB [8], wherein it now seems that the buffering capacity was overcome and acidification of the medium occurred. The rate of PTB hydrolysis was first order with respect to PTB and hydroxide but independent of phosphate concentration. The second order rate constant for hydrolysis of PTB in 50% methanol at 37° was 227 ± 11 M^{-1} sec^{-1} (N = 24). When PTB hydrolysis was studied by following changes in the absorbance spectrum under conditions where phosphate buffer was in 99-fold excess and the pH was maintained at 7.4, the rate of formation of the thiazine products was a pseudo first order process. The apparent first order rate constant k_{app} value in 2.5% methanol at 37° was $(2.6 \pm 0.1) \times 10^{-4}$ sec^{-1} and the chemical half-life was *ca.* 44 min. The rate constant was decreased in 50% methanol: $k_{app} = (5.7 \pm 0.3) \times 10^{-5}$ sec^{-1}. The hydrolysis of PTB was not reversible under physiological conditions.

Analysis of the reaction mixture of 10 mM PTB with 22 mM of 1-phenyl-1,2-propanedione (typical chromatographic analysis data are presented in Fig. 1b) gave evidence of α,β-dicarbonyl cleavage to benzoic acid (peak 3) and the concomitant cleavage fragment 1-hydroxyethyl-PTB (peak 4). PTB hydrolysis products (peak 2), benzoin (peak 6; cross-link formation), and 1-hydroxybenzyl-PTB fragment (cleavage of PTB adding to 1-phenyl-2-oxopropanone in the reverse orientation) were also formed (see Fig. 2). After 6 hr, there was 17% of PTB remaining and 37% of PTB had hydrolysed; there was therefore a maximum 46% of PTB engaged in cleavage reactions. The reaction progress had slowed, however, due to the loss of buffering and decrease in pH: the pH decreased more rapidly than for PTB hydrolysis to 6.0 after 400 min. When only benzoic acid is assayed as an indication of reaction progress, this slowing of reaction can be mistaken for reaction completion [8]. The pH of the reaction mixture decreased because of thiazolium hydrolysis and the formation of acid cleavage products. The rate constant $k_{Cleavage}$ value for the cleavage of 1-phenyl-1,2-propanedione by PTB at pH 7.4 and 37° was 0.010 ± 0.002 M^{-1} sec^{-1} (N = 3).

The concentration of α,β-dicarbonyl in advanced glycated proteins was determined so that the ratio of the rates of PTB hydrolysis and cleavage reactions *in vivo* could be estimated. Protein α,β-dicarbonyls were estimated in HSA and AGE-HSA (36 AGE-modified arginine and lysine residues per protein molecule) by derivatisation with [^{14}C]aminoguanidine which has been shown to bind α,β-dicarbonyl intermediates of glucose-derived advanced glycation [7]. HSA had 7.5 ± 1.7 mmol of α,β-dicarbonyls per mol of protein and AGE-HSA had 19.3 ± 2.5 mmol per mol of protein. For a concentration of HSA in blood plasma of 0.7 mM, the protein α,β-dicarbonyl concentration is predicted to be in the range 5.3–13.5 μM although the upper limit is an overestimation since even in diabetic patients the extent of modification of proteins by glucose-derived AGE was rarely greater than 3 mol per mol of protein [15]. Hence, under physiological conditions, the hydrolysis of PTB was favoured *ca.* 1000 times above that of cleavage of α,β-dicarbonyl compounds. Vasan *et al.* [8] were able to see evidence of the cleavage reaction because they used a concentration of α,β-dicarbonyl substrate *ca.* 2000-fold higher than the highest estimate of physiological concentration. Indeed, when PTB was incubated with physiological concentrations of α,β-dicarbonyl, only formation of the hydrolysis products was observed.

An alternative explanation for the cleavage of protein cross-links found previously [8] is the reduction of intermo-

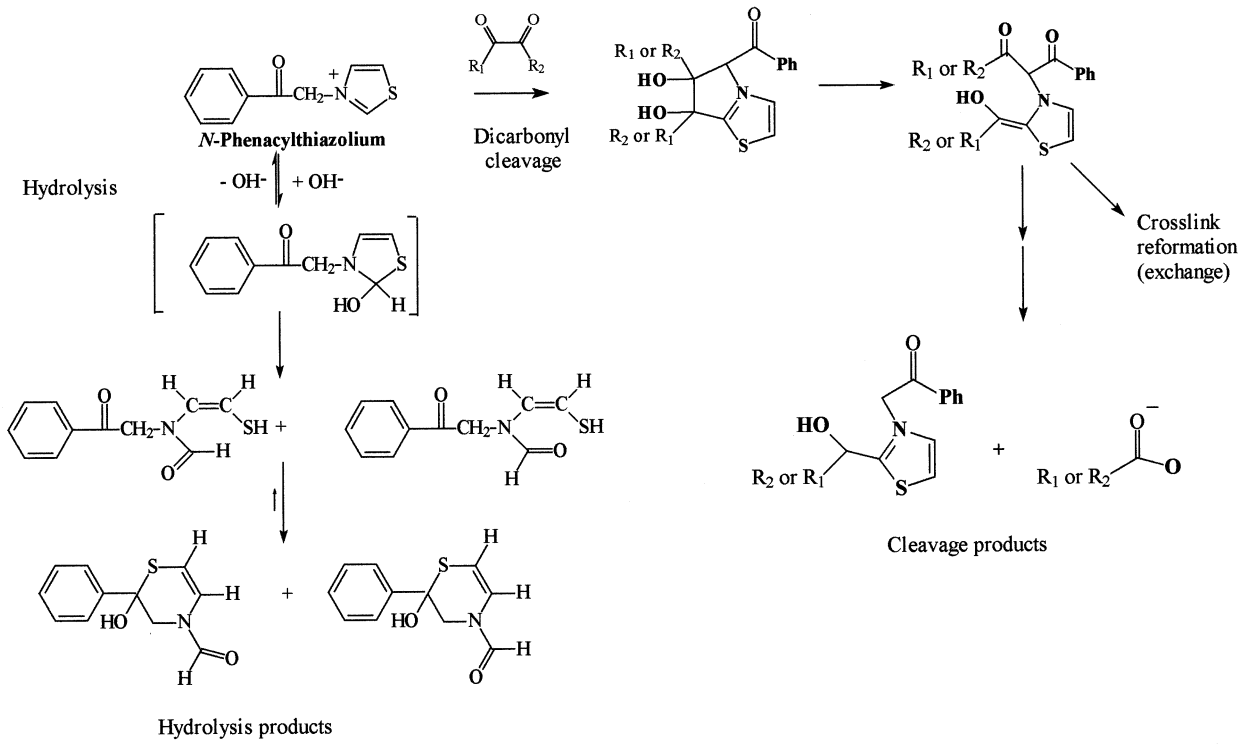

FIG. 2. Hydrolysis and α,β-dicarbonyl cleavage reactions of PTB. R_1 and R_2 are alkyl/aryl groups of cleavage reaction products from the α,β-dicarbonyl substrate; for asymmetrical α,β-dicarbonyl compounds, 4 cleavage products are expected.

lecular disulphide bonds by PTB hydrolysis products: AGE-HSA prepared *in vitro* consists of homodimer polypeptide fragments formed from oxidative degradation, linked by disulphide bonds [16]. To investigate evidence of this, we determined the thiol content of HSA and AGE-HSA incubated for 400 min with and without 3 mM of PTB in PBS at 37° and pH 7.4. The thiol content (HSA molar equivalents; mean ± SD, N = 3, *P* value with respect to corresponding incubation without PTB) was: HSA 0.46 ± 0.10, HSA + PTB 1.05 ± 0.29 ($P < 0.02$); AGE-HSA 0.33 ± 0.25, and AGE-HSA + PTB 0.75 ± 0.11 ($P < 0.05$). PTB therefore increased the thiol content of both HSA and AGE-HSA. Cleavage of disulphide bonds in AGE-HSA therefore may explain the apparent cross-link cleavage by PTB.

A further feature of thiazolium hydrolysis was the acidification. Experiments performed previously on advanced glycated albumin, collagen, and β-amyloid with 10–30 mM of PTB in PBS [8] exposed the proteins to PTB hydrolysis products and acidification, which may have slowed oxidative processes leading to cross-linking and changed protein conformation. Our attempts to repeat the previous findings of the interaction of PTB with α,β-dicarbonyl compounds have indicated several methodological problems with the study that question the validity of the conclusions. The rapid hydrolysis of PTB, interaction with physiologically relevant concentrations of α,β-dicarbonyl compounds, and susceptibility to medium acidification should be considered if the pharmacological effects of PTB are to be understood.

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