Scheme I

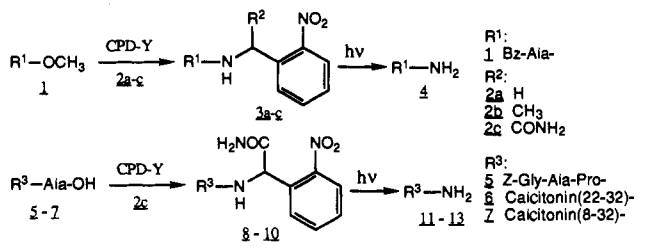

Table I. Enzymatic (CPD-Y) and Photochemical Transformations[a]

substrate	nucleophile	enzyme product (%)[b]	photochemical product (%)[c]
1	2a	3a (85)	4 (90)
1	2b	3b (25)	4 (95)
1	2c	3c (95)	4 (95)
5	2c	8 (95)	11 (95)
6[d]	2c	9 (91)	12[d] (95)
7[e]	2c	10 (90)	13[e] (95)

[a] Identification procedures were the following. **2b**, **2c**, and **3a-c**: elemental analyses, IR, ^1H and ^{13}C NMR. **5** and **8**: FAB MS, ^1H NMR. **6** and **7**: FAB MS, amino acid analysis. **9-13**: FAB MS, retention time, UV identical with those of authentic sample. [b] Transacylation: 70 nmol of enzyme (CPD-Y)/mmol of *N*-benzoylalanine methyl ester (**1**), nucleophile (**2a-c**) concentration 0.15-0.25 M in 5 mM EDTA; pH 8.0, 20 °C. Products (**3a-c**) were purified by HPLC. Transpeptidation: 1.0 μmol of CPD-Y/mmol of substrate (**5-7**), nucleophile (**2c**) concentration 0.2 M in 5 mM EDTA; pH 6.5, 20 °C. Products (**8-10**) were purified by HPLC. [c] Photolysis: peptide concentrations 1.0 mM, $NaHSO_3$ concentration 40 mM in 50% methanol (purged with nitrogen), pH adjusted with sodium hydroxide to 8.0 for peptides **3a-c** and 9.5 for **8-10**. Irradiation: 1-2 h, using a PS 200 Osram medium-pressure mercury lamp at a distance of 20 cm. A 40% copper sulfate solution was employed to filter out wavelengths below 320 nm. The peptide amides (**4**, **11**, **12**, and **13**) were purified by HPLC. [d] H-Pro-Gln-Thr-Ala-Ile-Gly-Val-Gly-Ala-Pro-X; **6**, X = Ala-OH; **12**, X = NH_2. [e] H-Leu-Gly-Thr-Tyr-Thr-Gln-Asp-Phe-Asn-Lys-Phe-His-Thr-Phe-Pro-Gln-Thr-Ala-Ile-Gly-Val-Gly-Ala-Pro-X; **7**, X = Ala-OH; **13**, X = NH_2. The substrate was chosen to resemble human calcitonin, but to avoid the methionine and the disulfide bridge.

partic acid α-amide as nucleophiles, which makes such peptide amides unavailable by this method.[5] e.g., calcitonin, which terminates in prolinamide. Furthermore, transpeptidation using ammonia as nucleophile has also led to peptide amides, albeit in very moderate yields.[5,6]

We now report an alternative method for the preparation of peptide amides, including those terminating in prolinamide, by transpeptidation with protected nucleophiles followed by photochemical deprotection.

The ability of carboxypeptidase-Y (CPD-Y, from yeast) to catalyze transacylation reactions of peptide esters[5] was used to prepare a series of model substances terminating in *N*-(2-nitrobenzyl)amido groups. Bz-Ala-OMe (**1**) was used as substrate and 2-nitrobenzylamine (**2a**) and (2-nitrophenyl)glycinamide (**2c**) were efficiently transferred by CPD-Y at pH 8.0, whereas 1-(2-nitrophenyl)ethylamine (**2b**) was not, being too strong a base to be efficient as a nucleophile at this pH (Scheme I).[7] The reaction is very efficient, and with **2a** and **2c** as nucleophiles, more than 95% of the substrate had disappeared within 60 min. The reaction with **2b** was less efficient (Table I). CPD-Y also serves as a very efficient catalyst for transpeptidation, especially for C-terminal alanine containing peptides.[8]

This was used to prepare the transpeptidation products with **2c** from Z-Gly-Ala-Pro-Ala-OH (**5**) and the human calcitonin fragment precursors calcitonin (22-32)-Ala-OH (**6**) and calcitonin (8-32)-Ala-OH (**7**) in high yields (Table I).

Subsequently irradiation with long-wavelength UV light (λ > 320 nm) was used to remove the 2-nitrobenzyl protective ligand[9] (Scheme I) to give the warranted peptide amides in high yield for substrates derived from **1**, **5**, **6**, or **7** (Table I). The photochemical step in the transformation (Scheme I) mimics the in vivo transformation of the ultimate amino acid in the peptide amide precursors, i.e., causes hydroxylation.[10] If the pH is kept sufficiently high, but not high enough to otherwise effect the peptide, the final hydrolysis takes place, again mimicking the in vivo process, thus making it an attractive alternative model for the α-amidating action of pituitary enzymes.[4,11]

The photolysis frequently resulted in the generation of colored byproducts which might function as inner filters. This problem could be eliminated by the addition of sodium hydrogen sulfite to the photolysis mixture as a scavenger.

In conclusion, we have shown a high-yield pathway for peptide amide formation from peptide precursors, which could be made available in large amounts by gene technology.

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Registry No. 1, 7244-67-9; **2a**, 1904-78-5; **2b**, 100311-54-4; **2c**, 138571-55-8; **3a**, 138571-56-9; **3b**, 138571-59-2; **3c**, 138571-60-5; **4**, 67711-04-0; **5**, 138571-57-0; **6**, 138571-58-1; **7**, 138605-26-2; **8**, 138605-27-3; **9**, 138605-28-4; **10**, 138605-29-5; **11**, 23927-09-5; **12**, 68801-56-9; **13**, 138605-30-8; CAD-Y, 9046-67-7.

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(7) Abbreviations used: Bz, *N*-benzoyl; Z, *N*-benzyloxycarbonyl; EDTA, ethylenediaminetetraacetic acid; HPLC, high-performance liquid chromatography; Other abbreviations of amino acids and peptides are according to guidelines of the IUPAC-IUB commission on Biochemistry Nomenclature.
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(9) Otherwise photolabile groups such as tryptophan, histidine, or tyrosine are not effected at wavelengths of >320 nm.[16a] No difference in yield or purity was observed at longer wavelength cutoff. 2-Nitrobenzoyl groups have previously been used in solid support synthesis of peptide amides as part of the linker which was photochemically cleaved to give peptide amides; cf. refs 3 and 10.
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In Situ Photoreactions of Proteins in Spectrometers Leading to Variations in Signal Intensities

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Takeda and Moriyama[1] have recently reported that the circular dichroism (CD) signal of a protein decreases with an increase in the measurement time. They have wondered whether this might be due to some photoreaction of the protein, since the changes occurred only in the presence of light and not in the dark. We show here that the phenomenon is general and that these CD changes indeed arise due to photochemical reactions involving some of the protein chromophores that absorb in the chosen wavelength region.

Figure 1A shows that irradiation[2] of bovine serum albumin (BSA) at 222 nm leads to alterations in some of its aromatic side

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(1) Takeda, K.; Moriyama, Y. *J. Am. Chem. Soc.* **1991**, *113*, 6700.
(2) We chose to use fluorescence as the method to monitor the photoreaction, since this technique is more sensitive than absorption or CD, particularly in detecting small amounts of the photoproducts. Irradiation was done using the 150-W Xenon lamp of a Hitachi F-4000 spectrofluorimeter, with its monochromator set at the chosen wavelength and the entrance slit band width at 20 nm. The amount of light incident at the center of the 1-cm quartz cuvette holding the protein solution in the sample compartment of the fluorimeter was estimated to be 0.5 mW/cm^2, or about 10^{14} photons/s. The intensity of 220-nm radiation incident on the sample in a JASCO J-20 CD instrument (450-W Xe lamp, 2-nm slit) was smaller and caused photodamage to a BSA solution that was 60% of what was seen when an identical solution was irradiated in the fluorimeter above.

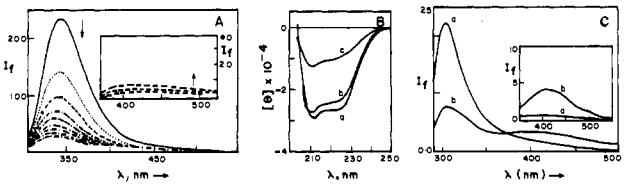

Figure 1. Panel A: Progressive change in the fluorescence spectra of BSA upon irradiation of the protein solution in aqueous buffer (2 h, monitored every 15 min, as the arrow indicates). Irradiation was done at 220 nm as per conditions given in ref 2. Excitation was at 295 nm for the main curves and 320 nm for the inset. I_f indicates fluorescence intensity in arbitrary units. Panel B: CD spectra of BSA before (a) and after irradiation in anaerobic (b) and aerobic (c) conditions, at 220 nm for 2 h. Ellipticity in the y axis is as deg·cm²·deciresidue/mol. CD measurements were done on a JASCO J 20 spectropolarimeter. Panel C: Fluorescence spectra of calmodulin solutions in aqueous buffer before (a) and after (b) irradiation at 220 nm for 2 h. Excitation was at 275 nm for the main curves and 320 nm for the inset.

chain moieties. The fluorescence due to the tryptophan (Trp) residues is progressively reduced, and a new weak emission band is generated in the 415-nm region. Such changes have been observed previously with several peptides and proteins,[3-5] generally upon irradiation of the ^1L_a,^1L_b band of Trp in the 280–300-nm (UVB) region, and the 415-nm emission has been attributed to *N*-formylkynurenine (NFK) and related products that were formed by the oxidation of the indole ring of Trp. Figure 1A shows that irradiation near 220 nm (the ^1B_a band in the UVC region) also produces the same results.

The conformation of the protein is altered in the process. CD spectra (Figure 1B) reveal substantial reduction in the secondary structure of BSA upon photolysis. Similar changes have been noted earlier[5] with a few other proteins. We find that photolysis of BSA denatured in 6 M guanidinium chloride does occur, but less efficiently than in the native form (as monitored by fluorescence, but with no accompanying CD changes, presumably since the unordered conformation of BSA in the denaturant solution is altered no further upon irradiation).

The photoreaction is sensitive to air. CD changes are far less pronounced when BSA is irradiated under anaerobic (N_2 flushed) conditions rather than in ambient aerobic conditions[3,6,7] (Figure 1B). It also depends on the amount of incident light: the losses in CD and fluorescence signals after 2 h of irradiation at 220 nm with spectral band widths of 20, 10, and 5 nm were about 35%, 15%, and 8%, respectively.

Tyr residues are also photolyzed. UVC irradiation of ribonuclease A reduces its Tyr fluorescence but not its CD. Irradiation of another Trp-free protein, calmodulin (CaM), at 220 nm for 2 h led to the loss of Tyr and the formation of a photoproduct that emits around 400 nm, which is very likely to be bityrosine.[8,9] Concimitantly, the secondary structure also changes from an initial 50% helix to 33% after the photoreaction. (The 400-nm emission was not seen with ribonuclease A photolysis. Bityrosine is apparently not formed here, probably because precursor Tyr residues are not appropriately disposed in this molecule as they are in CaM.[9])

Turning to the Phe residue, we irradiated (210 nm, 2 h) a synthetic peptide (comprising the first 26 residues of the sequence of the toxin pardaxin),[10] which contains three Phe residues and no Tyr or Trp, and found a small drop in both the emission and CD spectra, suggesting that Phe residues might also be affected by light.[3]

Photodamage depends on the aromatic residue content; Trp is known to be more photoreactive than Tyr, which in turn is more so than Phe.[3,7] This might explain the larger CD change seen in myoglobin,[1] which has 1.33 mol % Trp, while BSA has only 0.35 mol % Trp and ribonuclease A has no Trp at all.[11]

Interestingly, irradiation of a solution of α-helical poly(L-glutamic acid) with 220-nm light for 2 h brought about a 16% loss in the ellipticity value; somewhat similar results were also obtained with helical poly(L-lysine). This raises the possibility of the peptide chromophore itself being photosensitive, an issue that needs further study.

These results draw attention to the possibility of artifacts arising during spectral studies of protein kinetics (e.g., renaturation assays), which involve continuous spectral measurements in the UV region, though in most routine CD measurements (short-term illumination, 2-nm slits, and nitrogen flushing) these photoreactions may be insignificant and escape detection. Finally, protein photolytic damage can generate oxidation products and covalent aggregates,[12] a striking clinical manifestation of which occurs during light-induced forms of cataract on the eye lens.[13-15]

Registry No. Trp, 73-22-3; Tyr, 60-18-4; ribonuclease, 9001-99-4.

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(6) Anaerobic irradiation of Trp at <280 nm yields several photoproducts including kynurenine, while in the presence of air the key product is *N*-formylkynurenine (NFK).[3] Both kynurenine and NFK have very similar spectral features; see: Pileni, M. P.; Walrant, P.; Santus, R. *J. Phys. Chem.* **1976**, *80*, 1804.

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Direct Observation and Reactivity of Transient Ketenes Generated by Flash Photolysis

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Quantitative studies of ketene reactivities have concentrated on the hydration of a few transient species generated photolytically[1,2a,h-j] and on longer lived species that are usually heavily substituted.[2] Examples of the representative structural types RCH=C=O include ketene itself,[1a,2j] *n*-butylketene,[2a] phenyl-

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