p-Benzoyl-L-phenylalanine, A New Photoreactive Amino Acid

PHOTOLABELING OF CALMODULIN WITH A SYNTHETIC CALMODULIN-BINDING PEPTIDE*

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A new photoreactive amino acid analog, p-benzoyl-L-phenylalanine, is described. Convenient methods for the preparation of this amino acid and its subsequent incorporation into synthetic peptides by the solid-phase technique are outlined. To illustrate its utility, p-benzoyl-L-phenylalanine was substituted in place of tryptophan in a 17-residue calmodulin-binding peptide. The substitution did not measurably affect the affinity of this peptide for calmodulin. When this peptide was photolyzed at 350 nm in a 1:1 molar ratio with calmodulin in the presence of 500 μM CaCl₂, 70% of the calmodulin was derivatized. The specificity of the reaction was investigated by photolysis in the absence of CaCl₂ where little binding occurs; under these conditions little or no photolabeling occurred.

Photoaffinity labeling (1, 2) is a method which has been widely successful for the identification and localization of macromolecular receptors (3–5). Despite the general usefulness and importance of the technique for the labeling of peptide hormone receptors, there are no direct methods for the solid-phase synthesis of peptides containing photoactivatable probes. Photolabile peptides have generally been prepared by derivatization of the parent peptides with “heterobifunctional” cross-linking agents which combine in one molecule both chemically and photochemically reactive groups (6). The major disadvantage of this approach is that the photolabile group can only be introduced at chemically reactive sites present in the peptide, if indeed such sites exist. Furthermore, the covalent modification may limit the peptide’s abilities to bind and activate its receptor. An alternate approach involves the de novo synthesis of peptides containing derivatives of p-azidophenylalanine (6–9) which can form nitriles on photolysis. Such peptides are generally synthesized by solution rather than solid-phase methods because of the limited chemical stability of aryl azides. Another method involves the solid-phase synthesis of derivatives of p-azidophenylalanine–containing peptides which are subsequently converted to azides by multistep procedures (9). Finally, 3-[p-(trifluoromethyl)-1H-diazirin-3-yl]phenylalanine, a carbene precursor, has very recently been synthesized and may prove useful as a peptide photoaffinity label, although it has not yet been incorporated into peptides (10).

To synthesize the photoreactive peptides it would be desirable to prepare an amino acid which could be routinely incorporated into peptides by standard solid-phase techniques. Arylketone derivatives of phenylalanine appear to be ideal for this purpose because of their chemical stability and remarkably selective photochemistry. The n → π* transition of diarylketones can be effected with relatively low energy ultraviolet radiation giving rise to a triplet biradical which preferentially reacts with C–H bonds versus reaction with water (11, 25). Finally, in cases where benzophenone derivatives have been used for photolabeling they have been highly successful. Breslow (12) has photolyzed diarylketones attached to or complexed with hydrocarbons in an attempt to achieve selective functionalization of methylene groups remote from other functionalities. In a similar study, Biro and co-workers (13) demonstrated that arylketones react with C–H bonds with high regio- and stereospecificity when the ketones were photolyzed in crystalline surfactant hosts. 4-Benzyloxybenzylpentagastatin has also been shown to label bovine serum albumin (to which it binds, albeit with poor affinity) upon photolysis (14).

In this paper we describe the synthesis of p-benzoyl-L-phenylalanine (Bpa), and the incorporation of this residue into a 17-residue calmodulin-binding peptide by the solid-phase technique. Previously (15) we described the design, synthesis, and characterization of peptide I, which binds calmodulin (CaM) in a calcium-dependent manner with a 400 nM dissociation constant.

\[ \text{Lys-Leu-Xxx-Leu-Leu-Leu-Lys-Leu-Leu-Gly; peptide I, Xxx = Trp and peptide II, Xxx = Bpa. Based on model building (16, 26), we predicted that the tryptophanyl group at position 3 could be replaced by a Bpa residue without substantially changing the affinity of the peptide for CaM. Indeed, we have found that peptide II binds to CaM, and forms a covalent adduct upon photolysis.} \]

EXPERIMENTAL PROCEDURES

Materials

Bovine testes CaM was purchased from Pharmacia, and was homogeneous by SDS-polyacrylamide gel electrophoresis and reverse-phase HPLC. Peptide I was synthesized and purified as previously described (15).

\[ \text{Synthesis of Boc-t-Bpa} \]

\[ p\text{-Chloromethylbenzophenone—A solution of 190 g (0.51 mol) of } p\text{-methylbenzophenone (Aldrich), 130 ml of carbon tetrachloride, 98 g of sulfuryl chloride, and 55 mg of dibenzoyl peroxide was heated at reflux under nitrogen. At 2-h intervals, three additional 50 to 70-mg portions of dibenzoyl peroxide were added. After 18 h a final 50-mg portion of dibenzoyl peroxide was added. After 1 h, unreacted sulfuryl} \]

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† The abbreviations used are: Bpa, p-benzoyl-L-phenylalanine; CaM, calmodulin; SDS, sodium dodecyl sulfate; HPLC, high performance liquid chromatography; Boc, t-butoxycarbonyl; PBS, phosphate-buffered saline; EGTA, ethylenediaminetetraacetic acid.
chloride was removed by co-distillation with carbon tetrachloride (b.p. 69 °C). To the residue containing carbon tetrachloride, was added 5 g of potassium carbonate and 5 g of Woelm basic alumina. The mixture was refluxed for 15 min with stirring, and then filtered hot to give 57 g of crystals after cooling. Recrystallization from ethanol yielded 44.7 g (98%) of colorless crystals (m.p. 93-96 °C (17), m.p. 98 °C NMR 64.60 in CDCl₃). The product contained about 2% of 4-methylbenzophenone and 0.7% by-product dichloromethylbenzophenone (NMR 23.45 and 6.75, respectively).

N-Acetyl-L-α-cyano-p-benzoyl-DL-phenylalanine Ethyl Ester—A stirred mixture of 41.9 g (181 mmol) of p-chloromethylbenzophenone, 30.9 g (182 mmol) of ethyl acetoacetate, 173 g of anhydrous potassium carbonate, and 1.6 g of potassium iodide was refluxed overnight, cooled, and filtered. The solid was washed with acetone, and the combined filtrates were evaporated under reduced pressure. The residue was crystallized from 70 ml of ethanol by cooling to 5 °C. The resulting product (59 g) was dissolved in 900 ml of hot ethanol, treated with activated charcoal, filtered, and diluted with 900 ml of hexane to give 45.6 g (69.4%) of colorless crystals (m.p. 151-152 °C). Thin layer chromatography (Merck F-254 plates, 95/5, v/v, CHCl₃/HBOH) showed the product to be homogeneous (Rf 0.3), and its NMR was consistent with the structure.

C₆H₁₂NO₃
Calculated: C 69.44 H 5.50 N 4.50
Found: C 69.21 H 5.53 N 7.69

p-Benzoyl-DL-phenylalanine (dl-Bpa)—A suspension of 45.4 g of the above cyanoester in 188 ml of 80% aqueous acetonitrile was cooled to 5 °C. The resulting product (59 g) was dissolved in 900 ml of hot ethanol, treated with activated charcoal, filtered, and dried to give 36.1 g (95%) of DL-Bpa/HCl (m.p. 202-204 °C). The crude hydrochloride dissolved in 480 ml of boiling water was cooled on ice and the resulting fine solid was collected and washed with 80 ml of ethanol and dried to give 2.36 g (80%) of L-Bpa sesquihydrate (m.p. 178-179 °C, UV absorption maxima at 260 nm, ε max = 260,000 M⁻¹ cm⁻¹, ε min = 180 M⁻¹ cm⁻¹) in isopropyl alcohol.

C₁₆H₁₅NO₃·1.5 HzO
Calculated: C 64.85 H 6.12 N 4.73
Found: C 65.09 H 6.08 N 4.73

l-Bpa and N-Acetyl-p-benzoyl-DL-phenylalanine—A suspension of 6.24 g (20 mmol) of acetyl-DL-Bpa in 1.0 liter of water was stirred and 3.5 ml of 4 N H₂SO₄ was added to bring the pH to 7.5. The solution was filtered and 200 mg of aspergillus acidase I (Sigma) and 5 drops of toluene were added. The solution was stirred at 37 °C for 18 h and was cooled to 25 °C and filtered. The dämp solid product was dissolved in 50 ml of 0.5 N HCl at 70 °C. Celite was added, the suspension was filtered, and the cake was washed with hot water (50 ml). The clear filtrate was brought to pH 7 by the addition of 7.3 ml of 1 N NaOH. The solid was isolated by filtration (see below for filtration processing), washed with water and a small amount of ethanol, and dried to give 2.33 g (80%) of L-Bpa sesquihydrate (m.p. 178-179 °C, ε max = 3.0 ± 0.8 °, concentration 1.01 g/100 ml of 1 N HCl).

C₇H₁₄NO₃·1.5 H₂O
Calculated: C 68.28 H 6.28 N 3.79
Found: C 67.97 H 6.05 N 3.63

Synthesis and Purification of Peptide II—Peptide II was synthesized using the Merrifield method (18) using the protecting groups and synthetic protocol described for peptide I (15). The Bpa residue was incorporated using a 3-fold excess of the corresponding Boc-protected symmetric anhydride formed by reaction of 6 eq of Boc-L-Bpa with 3 eq of diisopropylcarbodiimide in CH₂Cl₂/CH₃OH/N,N-dimethylformamide (1/1/1) for 15 min at 0 °C. This was allowed to react with the resin and the coupling reaction was complete within 4 h as determined by the ninhydrin test (19). The peptide was cleaved from the resin by reaction with HF/p-cresol (10:1) at 0 °C for 60 min. The crude product (Fig. 14) was purified in a single step by reversed-phase HPLC using a Hamilton PRP-1 semipreparative column (purchased from Pierce), and a gradient of 35-41% aqueous acetonitrile containing 0.1% trifluoroacetic acid, at 0.35%/min and flow rate of 4.0 ml/min. Fractions containing pure peptide were pooled and lyophilized giving chromatographically homogeneous peptide in 15% overall yield based on the loading of the first amino acid in the resin.

Amino acid analysis (Leu=76) (8), Lys=82 (7), Glh=100 (1), Edman sequence analysis, and analytical reversed phase HPLC (Fig. 1B) showed that the desired peptide had been obtained in homogeneous form. Fast atom bombardment/mass spectroscopy gave the appropriate parent ion (M + H)⁺ = 2128 indicating that the Bpa residue had been preserved intact during the synthetic and purification procedures. This was confirmed by UV spectroscopy; peptide II showed a maximum single at 260 nm (ε = 18,000 M⁻¹ cm⁻¹) which is the same as that for Boc-Bpa in aqueous solution (ε max = 260, ε = 18,000 M⁻¹ cm⁻¹). Furthermore, the proton NMR spectrum of the peptide dissolved in dimethyl sulfoxide-d₆ was consistent with the proposed structure, and qualitatively similar to L-Bpa in the aromatic region. For l-Bpa the positions and primary resonance assignments of the protons at the ortho (o) and meta (m) positions of the phenylalanyl ring, and the ortho (o'), meta (m'), and para (p) positions of the benzoyl ring were: δ = 7.46 (parts/million from tetramethylsilane), doublet, J = 8 Hz, (2 protons, o); δ = 7.55, doublet of doublets, J = 8 Hz, (2 protons, m'); δ = 7.64, doublet, J = 8 Hz, (2 protons, o'); δ = 7.66, doublet, J = 8 Hz, (1 proton, p); δ = 7.71, doublet, J = 8 Hz, (2 protons, m). The assignments for the corresponding protons in peptide II were: δ = 7.48, doublet, J = 8 Hz, (2 protons, o); δ = 7.55, doublet of doublets, J = 8 Hz, (2 protons, m); δ = 7.63, doublet, J = 8 Hz, (2 protons, o'); δ = 7.64, doublet, J = 8 Hz, (1 proton, p'); δ = 7.69, J = 8 Hz, doublet (2 protons, m). Stock solutions of the peptide
RESULTS

Synthetic Operations

As described under "Experimental Procedures," DL-Bpa was synthesized in multigram quantities by a three-step synthetic procedure starting from commercially available 4-methybenzophenone (Scheme I).

Conversion of DL-Bpa to the acetyl derivative, and resolution by hydrolysis with aspergillus acylase I gave L-Bpa, which spontaneously precipitated from solution, and Ac-D-Bpa which remained soluble. The L-isomer was converted to its α-t-butylloxycarbonyl-protected derivative, and incorporated into peptide II by the standard solid-phase technique. No special precautions were found to be necessary in handling either Bpa-derivatives or Bpa-containing peptides, and they were found to be stable indefinitely when stored at -10 °C.

Noncovalent Complex Formation between CaM and Peptide II

Determination of the Stoichiometry of Binding—Polycrystallamide gel electrophoresis was previously used to show that peptide I forms a calcium-dependent 1:1 complex with calmodulin that is stable in the presence of 4 M urea (Fig. 2, lanes 1-5, and Ref. 15). Similarly, when CaM is incubated with 0.5 eq of peptide II in 0.5 mM CaCl₂, a new band with an intensity approximately equal to the calmodulin band was observed (Fig. 2, lane 7). When the peptide/CaM ratio was raised to (1:1), the CaM band disappeared and the band due to the complex increased in intensity (lane 8). No new bands were observed when the peptide/CaM ratio was 2:1 or 3:1 (lanes 9 and 10). In contrast, when the incubation and electrophoresis were carried out in the absence of calcium and with 1 mM EGTA added, no band attributable to a complex was formed, even when the peptide was in 3-fold excess over calmodulin (data not shown).

Binding Affinity of Peptide II for CaM—In order to verify that the substitution of L-Bpa for tryptophan in peptide II did not significantly alter the affinity of the peptide for calmodulin, the ability of peptides I and II to compete for calmodulin binding to the target enzyme myosin light chain

FIG. 1. Reverse-phase HPLC of crude and purified peptide II. The chromatographic conditions employed were: Hamilton PRP-1 analytical column, gradient of 9-54% aqueous acetonitrile containing 0.1% trifluoracetic acid α: 1%/min and a flow rate of 2 ml/min. A, 100 µg of crude product obtained from HF cleavage; B, 30 µg of peptide II after HPLC purification.

were stored frozen at -10 °C in aqueous solution at a concentration of 2 mg/ml. Under these conditions, it was stable for at least 6 weeks.

Photolabeling of Calmodulin—The photoreactions were carried out in polystyrene dishes (Costar 24-well dishes), with the wells uniformly positioned 1-2 cm from the light source (Rayonet Photochemical Reactor, 3500 A lamp, Southern New England Ultraviolet Co., with 9 lamps positioned horizontally). At the indicated times, samples were removed from the light source and stored at -20 °C until they were analyzed. Details of the HPLC analysis of photoreactions are described in the figure legends.

Miscellaneous—SDS-gel electrophoresis was performed with 12.5% polyacrylamide gels according to Laemmli (20) with 1.0 mM EGTA were analyzed. Details of the HPLC analysis of photoreactions are described in the figure legends.

NMR spectra were recorded using either a 300-MHz Nicolet NT or a General Electric QE 300 NMR spectrometer. Amino acid analyses were obtained using a Waters Associates Picotag system. Peptides were synthesized using a Beckman 990B synthesizer.

Myosin light chain kinase and myosin light chain kinase substrate were purified from chicken gizzards and kinase assays conducted as previously described (23, 27).
kinase was assayed. We have recently described the use of this competition assay to obtain reasonable estimates of the dissociation constants of peptides for calmodulin (23). Fig. 3 shows the activation of myosin light chain kinase induced by calmodulin in the absence and presence of 140 nM peptide I or peptide II. The activation curves in the presence of either peptide are identical within experimental error, and are markedly different than in the absence of inhibitory peptide. Previously it was shown that peptide I binds to CaM with a 0.4 (15) to 0.7 (23) nM dissociation constant. The identity of the curves for peptides I and II indicates that peptide II also binds calmodulin with a subnanomolar dissociation constant.

Photolabeling of CaM

Optimization of Conditions—In an initial attempt to optimize the conditions for photolabeling, CaM was irradiated for 6 min with 350-nm light at room temperature in the presence or absence of peptide II in various buffers, and the products separated by HPLC (Fig. 4). Without 350-nm radiation, a mixture of CaM and peptide II eluted as 2 distinct peaks with retention times of 13 and 16.5 min (panel A). A standard of CaM elutes as a single peak at 16.5 min (panel B). Thus, under the HPLC conditions employed, noncovalent complexes between CaM and peptide II are dissociated. In the absence of peptide II, irradiation of CaM had no effect on retention time, or peak height (not shown). When an equimolar mixture of CaM and peptide II was irradiated in phosphate-buffered saline (PBS, 20 mM sodium phosphate, 0.15 M NaCl, pH 7.0) containing 0.5 mM CaCl₂, the peak due to CaM decreased by 50% and a new broad peak with a retention time of 17.4 min appeared (panel C). Incubation of peptide II and CaM in PBS without CaCl₂ and with 1 mM EDTA resulted in a 25% decrease in the CaM peak indicating that less labeling had occurred. The yield of the presumed adduct at 17.4 min was very low, and several minor peaks were present (panel D). Previously we showed that the positively charged peptide I could interact in a nonspecific calcium-independent manner with CaM which is an acidic protein, and that this nonspecific binding is greatly reduced in the presence of 4 M urea (15). To test whether similar nonspecific interactions were giving rise to the small amount of calcium-independent labeling, the photolysis reactions were repeated in the presence of 4 M urea. In the presence of CaCl₂, the addition of urea yielded the same decrease of the CaM peak of approximately 50% as in the absence of urea (panel E), whereas without CaCl₂ and with 1 mM EDTA we observed less than a 10% decrease in the CaM peak and no new peaks were detected (panel F). Therefore, all further experiments were carried out in the presence of 4 M urea/PBS, containing 0.5 mM CaCl₂.

Time Course for Photolabeling—CaM was photolyzed with 1 eq of peptide II in PBS buffer containing 4 M urea and 0.5 mM CaCl₂, and aliquots removed at various times for analysis by HPLC (Fig. 5) or SDS-polyacrylamide gel electrophoresis (Fig. 6). The time course for the loss of CaM reached an asymptotic limit of 30% residual CaM at long exposure times, with a half-time of 4 min. The curve describing the appearance of peak II follows the disappearance of CaM, and also had a half-time of 4 min. The area of peak II at long exposure times corresponded to 50 ± 15% of the initial area of the calmodulin peak before photolysis. Thus, photolysis of CaM with 1 eq of peptide II leads to 70% labeling, and approximately 50% of the initial calmodulin can be isolated as a covalent complex.

FIG. 3. Inhibition of calmodulin-mediated activation of chicken gizzard myosin light chain kinase by peptide I and peptide II. The kinase activity (expressed as per cent of maximal activation) was measured as a function of the calmodulin concentration in the absence of added peptide (O—O) or in the presence of 140 nM peptide I (■—■) or peptide II (△—△).

FIG. 4. Ca**+-dependent photolabeling of CaM. Samples (200 μl) were chromatographed on a Hamilton PRP-1 semipreparative column equilibrated with 0.1% aqueous trifluoroacetic acid using a gradient of CH₃CN in the equilibrating buffer as shown above panel A. The flow rate was 4.0 ml/min. Panel A, 1400 pmol of CaM and 1400 pmol of peptide II in 20 mM sodium phosphate-buffered saline (0.15 M NaCl, pH 7.0), containing 0.5 mM CaCl₂; no exposure to 350-nm light. Panel B, 1400 pmol of CaM. Panel C, same as A except after 6 min exposure to 350-nm light. Panel D, same as C except CaCl₂ replaced by 1 mM EGTA. Panel E, same as C except with 4 M urea added. Panel F, same as D except with 4 M urea added.
buffer, pH 7.0, and 0.15 M NaCl in a total volume of 0.2 ml. Samples are the average of 2-4 experiments for each time point, and are of calmodulin, 0.5 mM CaCl₂, 4 M urea, 20 mM sodium phosphate tide

eluting in peak II. Increasing the molar ratio of peptide to CaM by sequential additions of fresh peptide solution resulted in a greater reduction in the CaM peak, but the chromatogram became more complex, presumably due to the formation of multivalent complexes.

The photolabeling reaction could be inhibited by addition of the non-photolabile peptide I, which competes with peptide II for binding to CaM. When peptide II and CaM in a 1:1 ratio were photolyzed for 10 min in the presence of a 5-fold excess of peptide I, a 20% decrease in the level of CaM was observed compared to a 70% reduction obtained in the absence of added peptide I.

Determination of the Stoichiometry of the CaM/Peptide II

**Photoadduct**—To further characterize the material eluting in peak II, a small amount of this material was purified by HPLC. A total of 9.2 nmol of CaM and 7.5 nmol of peptide II were photolyzed in PBS, 4 M urea, 0.5 M CaCl₂ for 10 min, and the products chromatographed under the conditions described in the legend to Fig. 4. Peak II was collected and lyophilized; the resulting product gave a single major peak on analytical HPLC with a small amount of calmodulin (10% of the adduct peak) as the only detectable impurity. The amino acid analysis of this material showed a large increase in leucine and lysine content over that of calmodulin alone, and was consistent with the incorporation of a single molecule of peptide II for each molecule of CaM. The compositional analysis for pure, unreacted CaM gave Leu₁⁷-Lys₁₄ (expected Leu₁⁹-Lys₁₄) and for the photoadduct yielded Leu₁⁶-Lys₁₄ which compares favorably to the values of Leu₁⁷-Lys₁₄ expected for a 1:1 CaM-peptide II complex.

**DISCUSSION**

*p*-Benzoylphenylalanine is a photoreactive amino acid which can easily be incorporated into a peptide by solid-phase synthesis. This amino acid was previously synthesized by R. Galardy (24), although the present work describes the first example of the incorporation of Bpa into peptides and its use as a photolabel. Substitution of Trp for Bpa in peptide I gave rise to a peptide which specifically labeled CaM in a calcium-dependent manner. Previously, we showed that the indole ring of the tryptophan in peptide I was directly involved in forming the CaM-peptide complex, and that it was held in a rigid, hydrophobic environment at the CaM-peptide interface (15). When Bpa was substituted for Trp, the peptide still formed a non-covalent complex of similar affinity; photolysis of this complex resulted in photolabeling of CaM with a 70% yield. Photolysis for longer periods failed to improve the yield, and the starting peptide disappeared from the chromatogram suggesting that a portion of the peptide either reacted intramolecularly or with solvent. The yield of labeled CaM could be increased by adding additional peptide, although this led to a complex product mixture resulting from a second low affinity peptide-binding site on CaM (15).

The ability to place Bpa anywhere in a peptide sequence provides an excellent method for mapping the binding sites of peptide receptors. By varying the position of Bpa in the sequence of various CaM-binding peptides, it should be possible to identify the residues involved in binding peptides and target enzymes.

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