Phenyl Azide Substituted and Benzophenone-Substituted Phosphonamides of 7-Methylguanosine 5′-Triphosphate as Photoaffinity Probes for Protein Synthesis Initiation Factor eIF-4E and a Proteolytic Fragment Containing the Cap-Binding Site†

Ashok J. Chavan,‡ Wojciech Rychlik,§ Dieter Blaas,∥ Ernst Kuechler,∥ David S. Watt,‡ and Robert E. Rhoads*,§

Departments of Chemistry and Biochemistry, University of Kentucky, Lexington, Kentucky 40536, and Institut für Biochemie der Universität Wien, Währinger Strasse 17, A-1090 Vienna, Austria

Received August 8, 1989; Revised Manuscript Received January 17, 1990

ABSTRACT: Three photoactive derivatives of the 7-methylguanosine-containing cap of eukaryotic mRNA were used to investigate protein synthesis initiation factor eIF-4E from human erythrocytes and rabbit reticulocytes. Sensitive and specific labeling of eIF-4E was observed with the previously described probe, [γ-^{32}P]-γ-[[(4-benzoylphenyl)methyl]amido]-7-methyl-GTP [Blaas et al. (1982) *Virology 116*, 339; abbreviated [^{32}P]BPM]. A second probe was synthesized that was an azidophenyltyrosine derivative of m^{7}GTP ([^{125}I]APTM), the monoanhydride of m^{7}GDP with [^{125}I]-*N*-(4-azidophenyl)-2-(phosphoramido)-3-(4-hydroxy-3-iodophenyl)propionamide. This probe allowed rapid and quantitative introduction of radioactivity in the last rather than the first step of synthesis and placed the radioactive label on the protein-proximal side of the weak P–N bond. A dissociation constant of 6.9 μM was determined for [^{125}I]APTM, which is comparable to the published values for m^{7}GTP. m^{7}GTP and APTM were equally effective as competitive inhibitors of eIF-4E labeling with [^{125}I]APTM. Like [^{32}P]BPM, [^{125}I]APTM labeled both the full-length (25 kDa) polypeptide and a 16-kDa degradation product, designated eIF-4E*, with labeling occurring in proportion to the amounts of each polypeptide present. A third probe, an azidophenylglycine derivative of m^{7}GTP ([^{32}P]APGM), the monoanhydride of m^{7}GDP with [^{32}P]-*N*-(4-azidophenyl)-2-(phosphoramido)acetamide, was also synthesized and shown to label eIF-4E specifically. Unlike [^{32}P]BPM and [^{125}I]APTM, however, [^{32}P]APGM labeled eIF-4E* approximately 4-fold more readily than intact eIF-4E. Tryptic and CNBr cleavage suggested that eIF-4E* consists of a protease-resistant core of eIF-4E that retains the cap-binding site and consists of approximately residues 47–182.

The entry of mRNA into the cycle of polypeptide chain initiation involves the recognition of the 7-methylguanosine-containing cap by a 25-kDa polypeptide, eukaryotic initiation factor (eIF)[1] 4E [reviewed in Rhoads (1988) and Sonenberg (1988)]. In animal cells, eIF-4E is isolated both in free form (Sonenberg et al., 1979; Hellmann et al., 1982) and in complexes with other polypeptides, one of which is termed eIF-4F (Tahara et al., 1981; Grifo et al., 1983; Edery et al., 1983). It is not clear whether initial recognition of mRNA is by free eIF-4E or by eIF-4F (Hiremath et al., 1989).

Chemical probes based on the cap structure have been useful in the elucidation of the function and mechanism of action of the eIF-4 group of polypeptides. Sonenberg and Shatkin (1977) used periodate-oxidized, cap-labeled mRNA and showed that several polypeptides in wheat germ postmitochondrial supernate or crude reticulocyte initiation factors were labeled following reduction with $NaBH_3CN$. Subsequently, Sonenberg (1981) showed that whereas only eIF-4E was cross-linked to this probe in the absence of ATP, the 46-kDa initiation factor eIF-4A was also cross-linked in the presence of ATP. The sensitivity of this type of probe was greatly enhanced by the introduction of ^{32}P instead of ^{3}H into the cap (Ray et al., 1985). A second type of probe, consisting of a short, capped oligonucleotide labeled at the 3′-terminus with [^{32}P]pCp, was used by Hellmann et al. (1982) and shown to bind reversibly to eIF-4E. A photoaffinity probe for cap-binding sites, the benzophenone-substituted analogue **1** ([^{32}P]BPM; Figure 1), was used to label the PB2 protein of influenza virus and cap-binding proteins in whole HeLa cell lysates (Blaas et al., 1982; Patzelt et al., 1983). A direct photo-cross-linking reaction was described by Pelletier and Sonenberg (1985), who used UV light to cross-link unmodified mRNA, labeled in the cap with ^{32}P, to crude reticulocyte initiation factors. These authors observed ATP-dependent labeling of eIF-4B and ATP-independent labeling of eIF-4E but no labeling of eIF-4A. Interactions between eIF-4E and the cap structure have also been studied by measuring the direct binding of m^{7}GDP, m^{7}GTP, and m^{7}G(5′)ppp(5′)G to the protein using fluorescence emission and circular dichroism (McCubbin et al., 1988, 1989; Carberry et al., 1989, 1990).

In the present work, we have explored the use of aryl azide derivatives of the cap as photoaffinity probes. Photolysis is

† This research was supported by Grants RO1 GM20818 and 1 FO6 TW01245 from the NIH (R.E.R.), Grant INT-8702295 from the NSF (R.E.R.), Grants RO1 GM35806 and SO7 RR05857 from the NIH (D.S.W.), and a grant from the Austrian Bundesministerium fur Wissenschaft und Forschung (D.B. and E.K.).

‡ Department of Chemistry, University of Kentucky.
§ Department of Biochemistry, University of Kentucky.
∥ Institut für Biochemie der Universität Wien.

[1] Abbreviations: Ar H, aromatic H; CDI, 1-cyclohexyl-3-(2-morpholinoethyl)carbodiimide metho-*p*-toluenesulfonate; eIF, eukaryotic initiation factor; HPLC, high-performance liquid chromatography; [^{125}I]APTM, monoanhydride of m^{7}GDP with [^{125}I]-*N*-(4-azidophenyl)-2-(phosphoramido)-3-(4-hydroxy-3-iodophenyl)propionamide; m^{7}G, 7-methylguanosine; [^{32}P]APGM, monoanhydride of m^{7}GDP with [^{32}P]-*N*-(4-azidophenyl)-2-(phosphoramido)acetamide; [^{32}P]BPM, [γ-^{32}P]-γ-[[(4-benzoylphenyl)methyl]amido]-m^{7}GTP; THF, tetrahydrofuran; SDS–PAGE, polyacrylamide gel electrophoresis in the presence of sodium dodecyl sulfate.

0006-2960/90/0429-5521$02.50/0

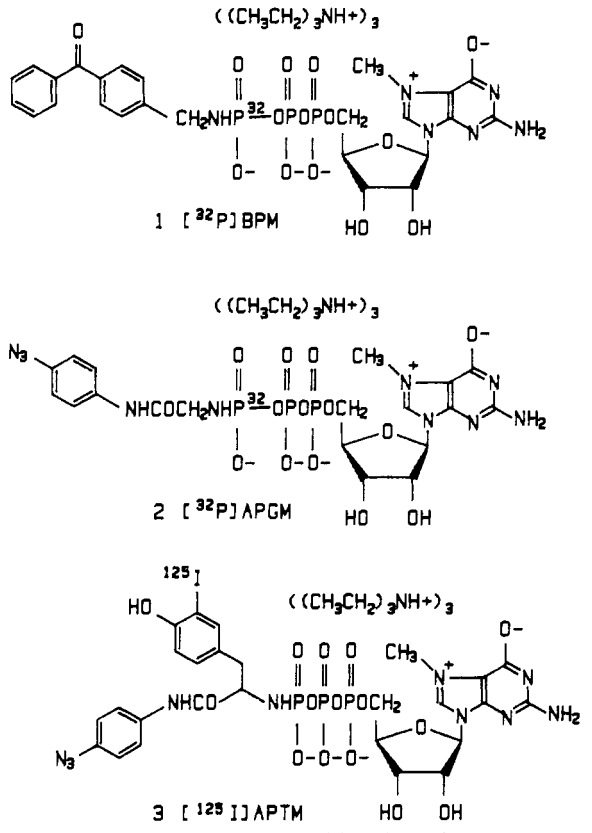

FIGURE 1: Photoaffinity probes used in this study.

expected to generate a singlet nitrene that would undergo ring expansion to a reactive dehydroazepine (Leyva et al., 1986; Shields et al., 1987), the species that would establish the covalent attachment to amino acid residues in the m^7GTP binding site. The requirements for an effective phenylazido-based photoaffinity probe initially suggested the development of the m^7GTP analogue **2** ([^{32}P]APGM; Figure 1) in which an aryl azide was linked to m^7GTP through a glycyl phosphonamide bond. This compound was shown in the present study to be a sensitive cap-binding site probe. However, the weak P–N bond in the phosphonamide linkage of [^{32}P]APGM would not survive the hydrolysis and chromatography conditions ultimately required for peptide purification and sequencing, a disadvantage shared by [^{32}P]BPM. This problem prompted the development of an alternate m^7GTP analogue **3** ([^{125}I]APTM), in which the aryl azide was linked to m^7GTP through a tyrosyl phosphonamide linkage. In the latter case, hydrolysis of the phosphonamide bond would not lead to loss of the radiolabel in the cross-linked protein. These two probes, [^{32}P]APGM and [^{125}I]APTM, were investigated and compared with [^{32}P]BPM in a study of the cap-binding site of eIF-4E and a derived proteolytic fragment.

Materials and Methods

Materials. Reagents were purchased from Aldrich (Milwaukee, WI) or Sigma (St. Louis, MO) and used directly in the reactions summarized below and in Figures 2 and 3. Radioisotopes were purchased from Amersham (Arlington Heights, IL) and New England Nuclear (Boston, MA). m^7GTP–Sepharose was purchased from Pharmacia LKB (Piscataway, NJ).

Analytical Methods. Elemental analyses were performed by Atlantic Microlabs (Atlanta, GA). Column chromatography was performed on Macherey Nagel Silica Gel 60. Preparative or analytical thin-layer chromatography on silica gel F254 was performed in 7:3 ethanol:1 M $(NH_4)HCO_3$. Ion-exchange chromatography was performed by using Bio-Rex 70 (Bio-Rad, Rockville Centre, NY).

[γ-^{32}P]m^7GTP. The method of Johnson and Walseth (1979) was used for the incorporation of 20 mCi of [^{32}P]-orthophosphate (carrier free) into 6 nmol of GTP. Incorporation was normally greater than 95%. At the conclusion of the synthesis, 36 nmol of nonradioactive GTP was added and the compound was converted to [γ-^{32}P]m^7GTP as described by Blaas et al. (1982). Specific activities were typically 150 Ci/mmol.

*Monoanhydride of m^7GDP with [^{32}P]-(4-Benzoylphenyl)-phosphoramidomethane ([^{32}P]BPM; **1**).* Condensation of [γ-^{32}P]m^7GTP with 4-(aminomethyl)benzophenone and purification were carried out as described by Blaas et al. (1982).

N-(4-Nitrophenyl)-α-(tert-butoxycarbamoyl)acetamide. To a solution of 2.0 g (14.5 mmol) of *p*-nitroaniline (**4**) in 20 mL of anhydrous THF were added 2.54 g (14.5 mmol) of *N*-(*tert*-butoxycarbamoyl)glycine and 3.11 g (15.1 mmol) of 1,3-dicyclohexylcarbodiimide. The mixture was stirred at 25 °C for 5 h, diluted with ethyl acetate, filtered, and concentrated to afford, after chromatography on silica gel, 1.4 g (33%) of *N*-(4-nitrophenyl)-α-(*tert*-butoxycarbamoyl)acetamide: mp 170–180 °C; IR (KBr) 3385, 3210, 3185, 3120, 3000, 2940, 2865, 1690, 1630, 1610, 1575, 1535 cm^{-1}; ^{1}H NMR (1:10 $CDCl_3$:DMSO-d_6) δ 1.47 (s, 9, $C(CH_3)_3$), 3.92 (d, J = 6 Hz, 2, CH_2), 6.16 (br s, 1, N*H*$COC(CH_3)_3$), 7.79 (d, J = 9 Hz, C-2 and C-6 Ar H), 8.13 (d, J = 9 Hz, 2, C-3 and C-5 Ar H), 10.17 (br s, 1, N*H*$COCH_2$).

*N-(4-Aminophenyl)-α-(tert-butoxycarbamoyl)acetamide (**5**).* A procedure (Smith et al., 1962) was repeated using 590 mg (2.0 mmol) of *N*-(4-nitrophenyl)-α-(*tert*-butoxycarbamoyl)acetamide and 1.34 g (24 mmol) of iron powder to afford, after column chromatography on silica gel using 1:48 methanol–ethyl acetate, 200 mg (38%) of **5**: mp 151–152 °C; IR (KBr) 3420, 3398, 3325, 3220, 3180, 3115, 2995, 2940, 1695, 1670, 1640, 1560, 1520 cm^{-1}; ^{1}H NMR (1:10 $CDCl_3$:DMSO-d_6) δ 1.43 (s, 9, $C(CH_3)_3$), 3.22 (br s, 2, NH_2), 3.73 (d, J = 6 Hz, 2, CH_2), 6.37 (br s, 1, N*H*$COC(CH_3)_3$), 6.50 (d, J = 8 Hz, 2, C-2 and C-6 Ar H), 7.18 (d, J = 8 Hz, 2, C-3 and C-5 Ar H), 9.16 (br s, 1, N*H*$COCH_2$); exact mass spectrum calcd for $C_{13}H_{19}N_3O_3$ 265.1428, found 265.1427.

N-(4-Azidophenyl)-α-(tert-butoxycarbamoyl)acetamide. To 2.65 g (10 mmol) of **5** in 25 mL of water, 20 mL of THF, and 4.5 mL of concentrated HCl was added 760 mg (11 mmol) of sodium nitrite in 2 mL of water at 0–5 °C. The mixture was stirred for 30 min and filtered, and the cold filtrate was treated with 715 mg (11 mmol) of sodium azide. The mixture was stirred for 20 min at 25 °C. The product was extracted with ethyl acetate, and the organic layer was washed with saturated sodium bicarbonate solution and brine and dried. The solvent was evaporated to afford 2.8 g (98%) of material sufficiently pure to be used in the next step. A sample of this material was recrystallized from ethyl acetate: mp 158–159 °C dec; IR (KBr) 2075, 1665, 1602 cm^{-1}; ^{1}H NMR ($CDCl_3$) δ 1.48 (s, 9, $C(CH_3)_3$), 3.93 (d, J = 6 Hz, 2, CH_2), 5.35 (br s, 1, CH_2N*H*), 6.97 (d, J = 9.2 Hz, 2, C-2 and C-6 Ar H), 7.51 (d, J = 8.6 Hz, 2, C-3 and C-5 Ar H), 8.37 (br s, 1, NHCO); exact mass spectrum calcd for $C_{13}H_{17}N_5O_3$ 291.1333, found 291.1329. Anal. Calcd for $C_{13}H_{17}N_5O_3$: C, 53.60; H, 6.21. Found: C, 54.52; H, 6.23.

*N-(4-Azidophenyl)-α-aminoacetamide (**6**).* To 1.0 g (3.4 mmol) of *N*-(4-azidophenyl)-α-(*tert*-butoxycarbamoyl)acetamide was added 10 mL of glacial acetic acid saturated with hydrogen chloride. The solution was allowed to stand for 30 min at 25 °C, diluted with anhydrous ether, and filtered to

afford 0.62 g (80%) of the hydrochloride of **6**: mp 190 °C dec; IR (KBr) 2104, 1664 cm^{-1}; ^1H NMR (DMSO-d_6) δ 3.79 (s, 2, CH_2), 7.17 (d, J = 11.2 Hz, 2, C-2 and C-6 Ar H), 7.68 (d, J = 11.2 Hz, 2, C-3 and C-5 Ar H), 8.31 (br s, 3, NH_3^+). Anal. Calcd for $C_8H_{10}N_5OCl$: C, 42.21; H, 4.43. Found: C, 42.20; H, 4.47. The hydrochloride salt of **6** was converted to the free amine **6** immediately before use by dissolution in a saturated sodium bicarbonate solution, extraction with ethyl acetate, and concentration.

Monoanhydride of m^{7}GDP with N-(4-Azidophenyl)-2-(phosphoramido)acetamide (APGM; 7). To 10 mg (15.9 μmol) of m^{7}GTP disodium salt in 0.7 mL of water at 10–15 °C was added 13.5 mg (31.8 μmol) of CDI during which the pH was maintained at 5.5–6.0 with HCl (Babkina et al., 1982). No appreciable rise in pH after adding 10 equiv of CDI (159 μmol) indicated that the m^{7}GTP was completely activated. To this solution was added 3.1 mg (16.2 μmol) of **6** in 150 μL of pyridine. The mixture was stirred for 4 h at 10–15 °C and purified on a DEAE cellulose column (30 × 2 cm) by using a linear 600-mL gradient from 50 to 700 mM triethylammonium bicarbonate buffer at a flow rate of 1 mL/min. Fractions 61–68 (fraction size = 5 mL) contained the desired product (R_f = 0.46 during chromatography on a silica gel plate) and unreacted m^{7}GTP. These fractions were combined and further purified on a SEP-PAK C-18 cartridge (Waters Associates) from which m^{7}GTP was eluted with 10 mL of 20 mM TEAB and the desired product was eluted with 10 mL of absolute methanol. The solvent was evaporated under reduced pressure at 20 °C to afford 6.8 mg (42%) of **7** as the tris(triethylammonium) salt: IR (KBr) 3420, 2140, 1720, 1650, 1260 cm^{-1}; ^{31}P NMR (D_2O) δ −1.00 (d, J = 14.3 Hz, 1, γP), −9.63 (d, J = 19.8 Hz, 1, αP), −20.67 (m, 1, βP) (Glonek et al., 1974; Babkina et al., 1975; Knorre et al., 1976). Ion-exchange chromatography afforded the trisodium salt: ^1H NMR (D_2O) δ 3.85 [s, 3, C-7 N(CH_3)], 5.80 (d, J = 2 Hz, 1, C-1′ H), 6.82 (d, J = 8 Hz, 2, Ar H), 7.34 (d, J = 8 Hz, 2, Ar H).

Monoanhydride of m^{7}GDP with [^{32}P]-N-(4-Azidophenyl)-2-(phosphoramido)acetamide ([^{32}P]APGM; 2). Condensation of [γ-^{32}P]m^{7}GTP (42 nmol) with **6** (30 μmol) and purification of the resulting [^{32}P]APGM were carried out as described by Blaas et al. (1982) for [^{32}P]BPM.

N-(4-Aminophenyl)-2-(tert-butoxycarbamoyl)-3-(4-hydroxyphenyl)propionamide. The procedure described above for the preparation of *N*-(4-nitrophenyl)-α-(*tert*-butoxycarbamoyl)acetamide was repeated using 0.52 g (4.8 mmol) of *p*-phenylenediamine (**8**), 1.5 g (5.3 mmol) of *N*-(*tert*-butoxycarbonyl)tyrosine, and 1.1 g (5.3 mmol) of 1,3-dicyclohexylcarbodiimide to afford *N*-(4-aminophenyl)-2-(*tert*-butoxycarbamoyl)-3-(4-hydroxyphenyl)propionamide that was sufficiently pure to be used directly in the next step. A sample was recrystallized from ethyl acetate–hexane: mp 95–97 °C; IR (KBr) 1690, 1660 cm^{-1}; ^1H NMR (acetone-d_6) δ 1.37 (s, 9, C(CH_3)$_3$), 2.92 (dd, J = 14, 8.2 Hz, 1, CH_2), 3.07 (dd, J = 14, 6 Hz, 1, CH_2), 4.39–4.50 (m, 1, CH), 6.60 (d, J = 8.5 Hz, 2, Ar H), 6.74 (d, J = 8.8 Hz, 2, Ar H), 7.09 (d, J = 8 Hz, 2, Ar H), 7.29 (d, J = 8.8 Hz, 2, Ar H), 8.85 (s, 1, OH); exact mass spectrum calcd for $C_{20}H_{25}N_3O_4$ 371.1846, found 371.1846.

*N-(4-Azidophenyl)-2-(tert-butoxycarbamoyl)-3-(4-hydroxyphenyl)propionamide (**9**).* The procedure described above for the preparation of *N*-(4-azidophenyl)-α-(*tert*-butoxycarbamoyl)acetamide was repeated using 1.9 g (5.1 mmol) of *N*-(4-aminophenyl)-2-(*tert*-butoxycarbamoyl)-3-(4-hydroxyphenyl)propionamide, 390 mg (5.6 mmol) of sodium nitrite, and 364 mg (5.6 mmol) of sodium azide to afford, after chromatography on silica gel using 1:1 hexane–ethyl acetate, 1.24 g (61%) of **9**: mp 85–86 °C; IR (KBr) 2120, 1680, 1620 cm^{-1}; ^1H NMR (acetone-d_6) δ 1.37 (s, 9, C(CH_3)$_3$), 2.92 (dd, J = 14, 8.2 Hz, 1, CH_2), 3.07 (dd, J = 14, 6 Hz, 1, CH_2), 4.40–4.54 (m, 1, CH), 6.19 (br d, 1, CHN*H*), 6.75 (d, J = 8.5 Hz, 2, Ar H), 7.01 (d, J = 8.8 Hz, 2, Ar H), 7.11 (d, J = 8.5 Hz, 2, Ar H), 7.66 (d, J = 8.8 Hz, 2, Ar H), 8.18 (s, 1, CONH), 9.32 (s, 1, OH); exact mass spectrum calcd for $C_{20}H_{23}N_5O_4$ 397.1750, found 397.1752.

*N-(4-Azidophenyl)-2-amino-3-(4-hydroxyphenyl)propionamide (**10**).* The procedure described for the preparation of **6** was repeated using 2 g (5.0 mmol) of **9** and 20 mL of glacial acetic acid saturated with hydrogen chloride gas to afford 726 mg (45%) of the hydrochloride salt of **10**: IR (KBr) 2120, 1680, 1615 cm^{-1}; ^1H NMR (DMSO-d_6) δ 2.90–3.16 (m, 2, CH_2), 4.05–4.20 (m, 1, CH), 6.69 (d, J = 8.4 Hz, 2, Ar H), 7.07 (d, J = 3.6 Hz, 2, Ar H), 7.11 (d, J = 4 Hz, 2, Ar H), 7.62 (d, J = 8.8 Hz, 2, Ar H), 8.31 (br s, 3, NH_3^+), 9.36 (br s, 1, CONH), 10.80 (s, 1, OH); exact mass spectrum calcd for $C_{15}H_{16}ClN_5O_2 - N_2$ 305.0931, found 305.0931.

*Monoanhydride of m^{7}GDP with N-(4-Azidophenyl)-2-(phosphoramido)-3-(4-hydroxyphenyl)propionamide (APTM; **11**).* The procedure described for the preparation of **7** was repeated using 10 mg (16 μmol) of m^{7}GTP disodium salt, 68 mg (160 μmol) of CDI, and 5.2 mg (18 μmol) of **10** (as the free base) to afford 6.8 mg (38%) of **11** as the tris(triethylammonium) salt: R_f = 0.52 during chromatography on a silica gel plate; IR (KBr) 3366, 2937, 2738, 2491, 2120, 1616, 1507, 1476 cm^{-1}; ^{31}P NMR (D_2O) δ −3.80 (d, J = 19 Hz, 1, γP), −10.91 (d, J = 19 Hz, 1, αP), −22.06 (t, J = 19 Hz, 1, βP) (Glonek et al., 1974; Babkina et al., 1975; Knorre et al., 1976). Ion-exchange chromatography afforded the trisodium salt: ^1H NMR (D_2O) δ 3.80 (s, 3, C-7 N(CH_3)), 5.84 (d, J = 2 Hz, 1, C-1′ H), 6.76 (d, J = 8 Hz, 2, Ar H), 6.82 (d, J = 8 Hz, 2, Ar H), 7.12 (d, J = 8 Hz, 2, Ar H), 7.18 (d, J = 8 Hz, 2, Ar H).

*N-(4-Azidophenyl)-2-(tert-butoxycarbamoyl)-3-(4-hydroxy-3-iodophenyl)propionamide (**12a**) and N-(4-Azidophenyl)-2-(tert-butoxycarbamoyl)-3-(4-hydroxy-3,5-diiodophenyl)propionamide (**12b**).* To a solution of 500 mg (1.26 mmol) of **9** and 226 mg (1.51 mmol) of sodium iodide in 4.5 mL of acetonitrile and 0.9 mL of water at 0 °C was added 180 μL (1.51 mmol, 1.2 equiv) of *tert*-butyl hypochlorite (Kometani et al., 1985) dropwise. The solution was stirred for 30 min at 0 °C and diluted with 50 mL of ethyl acetate. The organic solution was washed successively with 5% sodium thiosulfate solution and brine, dried, and concentrated to afford 703 mg of crude product. This was chromatographed on silica gel using 5:1 dichloromethane–ethyl acetate to afford 105 mg (16%) of **12a**: mp 188–189 °C dec; IR (KBr) 2120, 2100, 1680, 1670 cm^{-1}; ^1H NMR (DMSO-d_6) δ 1.32 (s, 9, C(CH_3)$_3$), 2.74 (dd, J = 14, 8 Hz, 1, CH_2), 2.88 (dd, J = 14, 5.2 Hz, 1, CH_2), 4.16–4.30 (m, 1, CH), 6.76 (d, J = 8.8 Hz, 2, Ar H), 6.96–7.14 (m, 3, Ar H), 7.60–7.70 (m, 3, Ar H); ^{13}C NMR (DMSO-d_6) δ 28.14, 56.69, 78.10, 84.18 (CI), 114.44, 119.39, 120.73, 130.36, 130.45, 133.86, 136.13, 139.10, 155.03, 155.37, 170.64; exact mass spectrum calcd for $C_{20}H_{22}IN_5O_4 - N_2 + H_2$ 497.0812, found 497.0785. In addition, 317 mg (39%) of **12b** was isolated: mp 105–106 °C; IR (KBr) 2100, 1690, 1670, 1600 cm^{-1}; ^1H NMR (acetone-d_6) δ 1.37 (s, 9, C(CH_3)$_3$), 2.90 (dd, J = 14, 8 Hz, 1, CH_2), 3.13 (dd, J = 14, 5.2 Hz, 1, CH_2), 4.42–4.54 (m, 1, CH), 6.29 (br s, 1, CHN*H*), 7.05 (d, J = 8.9 Hz, 2, Ar H), 7.69 (d, J = 8.7 Hz, 2, Ar H), 7.72 (s, 2, Ar H), 7.94 (br s, 1, CONH), 9.41

(br s, 1, OH); ^{13}C NMR (acetone-d_6) δ 28.51, 36.10, 57.32, 79.55, 84.17 (CI), 120.10, 121.94, 122.04, 134.72, 135.83, 136.87, 141.24, 156.27, 170.69; exact mass spectrum calcd for $C_{20}H_{21}I_2N_5O_4 - N_2 + H_2$ 622.9779, found 622.9753.

*Monoanhydride of m^{7}GDP Tris(triethylammonium) Salt with N-(4-Azidophenyl)-2-(phosphoramido)-3-(4-hydroxy-3-iodophenyl)propionamide (**13**).* The procedure for the preparation of **6** was repeated using 30 mg (60 μmol) of **12a** and 5 mL of glacial acetic acid saturated with hydrogen chloride gas. The solution was stirred for 30 min, with adjustment of the pH to 7 by the addition of saturated sodium bicarbonate solution, extracted with ethyl acetate and concentrated to afford 21 mg (87%) of *N*-(4-azidophenyl)-2-amino-3-(4-hydroxy-3-iodophenyl)propionamide: IR (KBr) 3280, 3070, 2100, 1675, 1605 cm^{-1}.

The procedure described for the preparation of **7** was repeated using 10 mg (16 μmol) of m^{7}GTP disodium salt, 81.6 mg (192 μmol) of CDI, and 18 mg (42 μmol) of *N*-(4-azidophenyl)-2-amino-3-(4-hydroxy-3-iodophenyl)propionamide to afford 5.1 mg (28%) of **13** as the tris(triethylammonium) salt (R_f = 0.65 on silica gel plate): IR (KBr) 3300, 2960, 2120, 1720 cm^{-1}; ^{31}P NMR (D_2O) δ −3.78 (d, J = 13.5 Hz, 1, γP), −10.89 (d, J = 15.6 Hz, 1, αP), −22.07 (m, 1, βP) (Glonek et al., 1974; Babkina et al., 1975; Knorre et al., 1976). Ion-exchange chromatography afforded the trisodium salt: ^1H NMR (D_2O) δ 3.85 (s, 3, C-7 N(CH_3)), 5.85 (d, J = 2.3 Hz, 1, C-1′ H), 6.59 (d, J = 8.3 Hz, 1, Ar H), 6.85 (d, J = 8.8 Hz, 2, Ar H), 7.02 (dd, J = 8.3, 2.1 Hz, 1, Ar H), 7.16 (d, J = 8.8 Hz, 2, Ar H), 7.46 (d, J = 2.1 Hz, 1, Ar H).

*Monoanhydride of m^{7}GDP with [^{125}I]-N-(4-Azidophenyl)-2-(phosphoramido)-3-(4-hydroxy-3-iodophenyl)-propionamide and [^{125}I]-N-(4-Azidophenyl)-2-(phosphoramido)-3-(4-hydroxy-3,5-diiodophenyl)propionamide ([^{125}I]APTM; **3**).* Two procedures were used for radioiodination of **11** to give [^{125}I]APTM. In the first, to 6 μL of Na^{125}I (3 mCi, 1.3 nmol) in a Reacti-vial (Pierce Chemical Co., Rockford, IL), were added 6 μL of a solution of 41.9 nmol of **11** in 1 M potassium phosphate buffer, pH 7.5, and 7.8 μL (5.2 nmol) of a solution of *tert*-butyl hypochlorite (Kometani et al., 1985) in acetonitrile. The mixture was allowed to stand at 25 °C for 1 h with occasional vortex stirring. The reaction was quenched by adding 10 μL of 5% aqueous sodium thiosulfate solution. Chromatography on a silica gel plate and autoradiography confirmed the presence of the desired product (R_f = 0.60). Typically 50% of the iodine was incorporated into the product. The mixture was purified on a C-18 SEP-PAK cartridge from which the unreacted radioiodide was eluted with 20 mL of water and the desired probe [^{125}I]APTM was eluted with absolute methanol. The specific activity of [^{125}I]APTM was 2–5 Ci/mmol. The second method employed 1,3,4,6-tetrachloro-3α,6α-diphenylglycoluril (IODO-GEN; Pierce) and followed the procedure of Markwell and Fox (1978). IODO-GEN was dissolved in chloroform at a concentration of 2 mg/mL, and 5 μL was pipetted into an Eppendorf tube. The chloroform was dried with a N_2 stream, and into the tube was pipetted 2 μL of 1 M sodium phosphate buffer, pH 7.5, 3 μL (193 nmol) of a solution of **11** in 50% aqueous ethanol, and 5 μL of Na^{125}I (0.5 mCi, 2.3 pmol). After 10 min, the solution was pipetted out and used without purification in photoaffinity labeling experiments. Chromatography on a silica gel plate indicated complete incorporation of ^{125}I into APTM. From the amount of reactants used, one can calculate that only 0.001% of **11** was converted into [^{125}I]APTM, yielding a specific activity of 2.5 Ci/mmol; higher specific activities could be achieved by using more Na^{125}I.

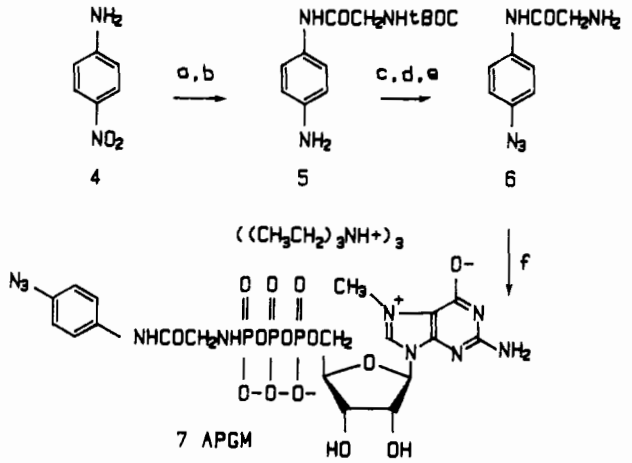

FIGURE 2: Synthesis of APGM and [^{32}P]APGM. Individual steps in the synthesis are (a) tBOCGly, DCC; (b) Fe, HOAc; (c) $NaNO_2$, HCl followed by NaN_3; (d) HCl gas, HOAc; (e) aqueous $NaHCO_3$; and (f) CDI and m^{7}GTP (to give **7**) or [γ-^{32}P]m^{7}GTP (to give **2**).

Purification of eIF-4E and eIF-4E.* Rabbit reticulocyte postribosomal supernate and human erythrocyte lysate were used to prepare eIF-4E (Webb et al., 1984; Rychlik et al., 1986). Protein concentration was estimated by the method of Bradford (1976). The 16-kDa proteolytic fragment (eIF-4E*) was separated from eIF-4E by HPLC using conditions described previously (Rychlik et al., 1986) except that the linear gradient of acetonitrile was 0–60%.

Affinity Labeling of eIF-4E. The standard affinity labeling reaction mixture was 25 μL and contained 20 mM Hepes-KOH, pH 7.6, 0.2 mM EDTA, 100 mM KCl, 2 mM $MgCl_2$, eIF-4E at 160 μg/mL, and varying amounts of radioactive mixed with nonradioactive affinity probe. Reaction mixtures containing [^{32}P]APGM or [^{125}I]APTM were irradiated in Eppendorf tubes standing in an ice–water mixture with a Vetter GmBH hand-held UV lamp (40 W, type UVKL-6U) placed directly over the tubes. Maximal incorporation of radioactivity occurred when reaction mixtures were allowed to stand 5 min before irradiation and then irradiated for 15 min. Irradiation of reaction mixtures containing [^{32}P]BPM was performed as described previously (Blaas et al., 1982). Protein was precipitated from reaction mixtures with 80% aqueous acetone, collected by centrifugation, and dissolved at room temperature for 30 min in 10 μL of sample buffer (Laemmli & Favre, 1973). (Heating of samples at this stage caused considerable loss of covalently bound radioactivity in the case of [^{32}P]BPM and [^{32}P]APGM.) Electrophoresis was performed as previously described (Laemmli & Favre, 1973). Gels were dried and exposed to Kodak XAR film with intensifying screens at −70 °C. Bands were excised from dried gels and radioactivity was determined in a scintillation spectrometer.

Protein Cleavage. eIF-4E (200 μg) was reduced and carboxymethylated as described previously and subjected to reverse-phase HPLC (Rychlik et al., 1986). Peaks corresponding to eIF-4E and eIF-4E*, as determined by SDS–PAGE, were collected and individually subjected to CNBr cleavage as described by Allen (1981). eIF-4E* was digested with trypsin and the peptides were resolved by reverse-phase HPLC as described previously (Rychlik et al., 1987a,b).

Results

Three photoaffinity probes containing the m^{7}GTP moiety were used to study the cap-binding site of eIF-4E (Figure 1). The reaction of [^{32}P]BPM with eIF-4E is shown in Figure 4. Labeling of the 25-kDa eIF-4E was detectable even at nano-

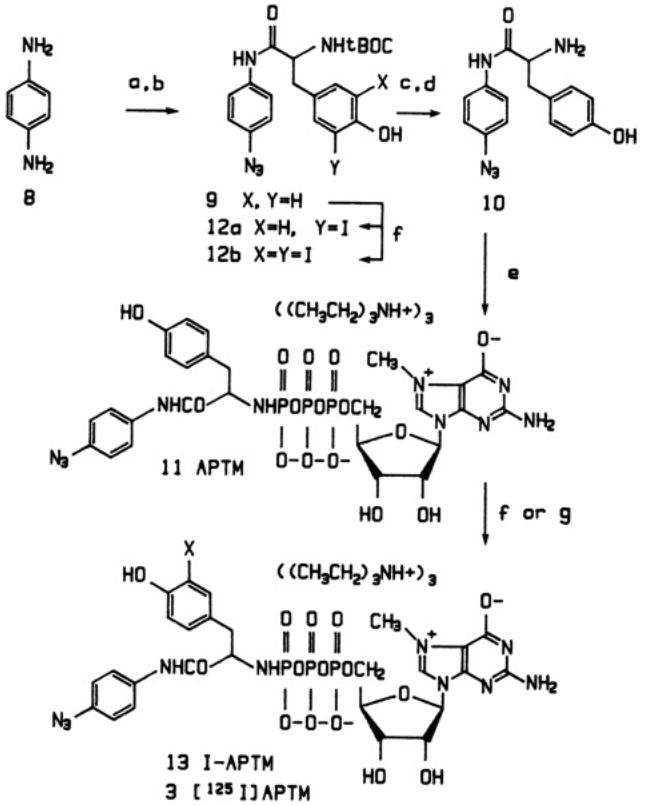

FIGURE 3: Synthesis of APTM and derivatives. Individual steps in the synthesis are (a) tBOCTyr, DCC; (b) $NaNO_2$, HCl followed by NaN_3; (c) HCl gas, HOAc; (d) aqueous $NaHCO_3$; (e) CDI, m^7GTP; (f) NaI, tBuOCl; and (g) [^{125}I]NaI, tBuOCl.

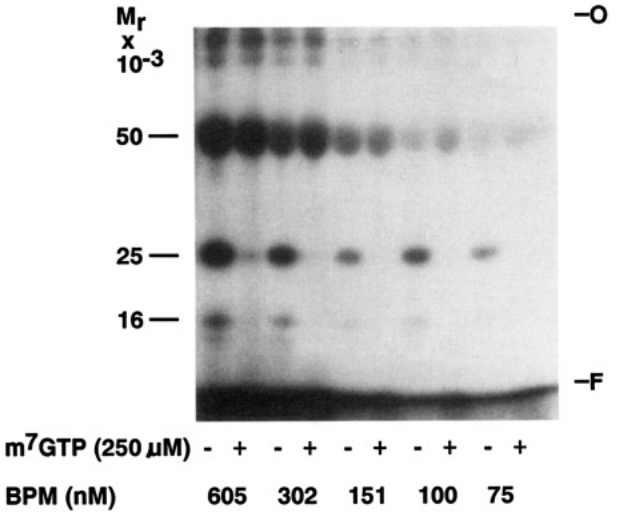

FIGURE 4: Photoaffinity labeling of eIF-4E with [^{32}P]BPM. Labeling was performed in 25-μL reactions containing 160 ng of a partially purified preparation of rabbit reticulocyte eIF-4E as described under Materials and Methods. Varying amounts of [^{32}P]BPM and m^7GTP were used as indicated. Separation was by SDS–PAGE on 12.5% gels. O, origin; F, dye front.

molar concentrations of [^{32}P]BPM. The labeling was inhibited by m^7GTP while that of an unidentified 50-kDa protein was not. A second polypeptide of approximately 16 kDa was also specifically labeled by [^{32}P]BPM. This polypeptide has been observed previously in eIF-4E preparations from human erythrocyte (Rychlik et al., 1986) and HeLa cell (Buckley & Ehrenfeld, 1986) and is here designated eIF-4E*.

A disadvantage of [^{32}P]BPM is the acid lability of the P–N bond. Attempts to purify [^{32}P]BPM-labeled eIF-4E led to significant loss of the radiolabel, even at neutral pH (data not shown). In order to incorporate a radiolabel in such a way

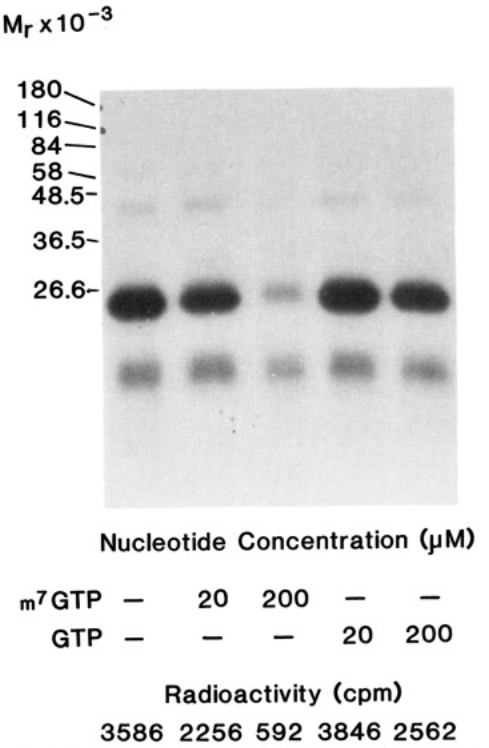

FIGURE 5: Labeling of eIF-4E with [^{125}I]APTM. The standard affinity labeling reaction mixture (see Materials and Methods) contained 220 μg/mL eIF-4E, 20 μM [^{125}I]APTM (1.28 Ci/mmol), and the additional nucleotides indicated. Irradiation was for 10 min at 0 °C. Samples were separated on 12.5% SDS–PAGE and the gel was dried and subjected to autoradiography for 10 h. Bands were excised and counted by Cerenkov radiation. The mobilities of standard proteins are given.

that P–N bond hydrolysis would be inconsequential following cross-linking, we introduced an ^{125}I radiolabel into the azide-containing portion of a different probe, APTM. As shown in Figure 3, the coupling of **10** to m^7GTP led to the nonradiolabeled compound **11**. The direct iodination of **11** provided an inseparable mixture of the mono- and diiodinated products as well as unreacted **11**. In order to characterize these products individually, the iodination products **12a** and **12b**, which were derived from the urethane **9**, were separated and, in the monoiodinated case, converted to the m^7GTP analogue **13**, which was fully characterized. Radioiodination of **11** provided [^{125}I]APTM (**3**), which was stable for extended periods at −20 °C in absolute methanol (less than 10% decomposition in a month).

[^{125}I]APTM was an effective photoaffinity label for eIF-4E (Figure 5). When [^{125}I]APTM was used at a concentration of 20 μM in the labeling reaction, m^7GTP at the same concentration reduced incorporation by almost half while 200 μM m^7GTP nearly eliminated incorporation. GTP at 20 μM actually stimulated cross-linking slightly, an effect reported previously for the noncovalent binding of capped oligonucleotides to eIF-4E (Hellmann et al., 1982), whereas 200 μM GTP inhibited incorporation 50% due to photoocclusion. Figure 5 also shows that [^{125}I]APTM labeled eIF-4E*. Comparison of this autoradiogram with the stained gel (not shown) indicated that the intensity of labeling was in proportion to the relative amounts of eIF-4E and eIF-4E*.

The binding of [^{125}I]APTM to eIF-4E was further investigated as a function of ligand concentration (Figure 6). The binding curve indicated saturation of a site on the protein (panel A). A Scatchard plot (Freifelder, 1982) indicated a single type of binding with an apparent K_d of 13.5 μM (panel B). However, the x-intercept indicated that at infinite con-

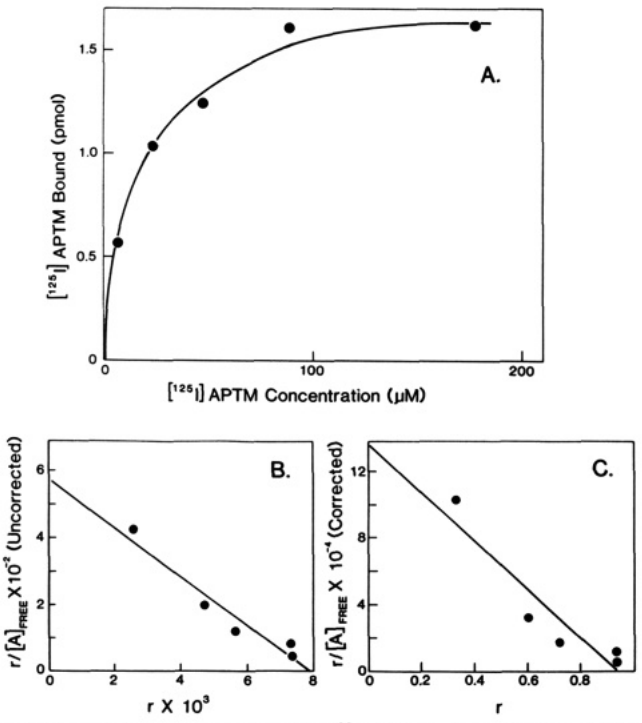

FIGURE 6: Titration of eIF-4E with [^{125}I]APTM. The standard affinity labeling reaction was carried out as described under Materials and Methods by using 220 μg/mL purified human erythrocyte eIF-4E and the amounts of [^{125}I]APTM (5 Ci/mmol) indicated. (A) Saturation curve; (B) Scatchard analysis (Freifelder, 1982); (C) corrected Scatchard analysis (see Results).

centration of [^{125}I]APTM, only 0.0078 mol of [^{125}I]APTM was incorporated per mole of protein, reflecting the competitive reaction of the active intermediate with water or other components. To correct for the efficiency of cross-linking, each value of bound [^{125}I]APTM was normalized by dividing by 0.0078. When the Scatchard plot was redrawn with the normalized values, a K_d of 6.9 μM was obtained (panel C), in better agreement with the K_d of 3.7 μM reported by Carberry et al. (1989) for m^{7}GTP.

To examine the specificity of [^{125}I]APTM binding to eIF-4E, we photolyzed reaction mixtures containing 2 μM [^{125}I]APTM in the presence of increasing amounts of either m^{7}GTP (Figure 7A) or APTM (Figure 7B). In both cases, labeling of eIF-4E was reduced in the presence of the competitor. The ratio of the dissociation constant of [^{125}I]APTM to that of each competitor was determined graphically (Ofengand and Henes, 1969) to be 1.6 for m^{7}GTP (Figure 7C) and 1.5 for APTM (Figure 7D). The deviation of these values from 1.0, especially in the case where [^{125}I]APTM competed with APTM, reflects either the nonequilibrium nature of photoaffinity labeling or a slightly poorer binding of the iodinated probe. However, the fact that the ratio was essentially the same for the two competitors indicated that APTM bound to eIF-4E with the same affinity as m^{7}GTP.

A third probe for eIF-4E, [^{32}P]APGM (**2**), combined the phenylazido moiety of APTM with a spacer arm that avoided the iodotyrosyl group and was more similar in steric bulk to [^{32}P]BPM. When [^{32}P]APGM was tested as a photoaffinity probe for eIF-4E, an unexpected result occurred: labeling of the 16-kDa degradation product, eIF-4E*, occurred preferentially to that of the intact 25-kDa eIF-4E (Figure 8B). Coomassie staining of the protein preparation used in the experiment demonstrated that eIF-4E was in considerable abundance over eIF-4E* (Figure 8A), but at low concentrations of [^{32}P]APGM, essentially only eIF-4E* was labeled

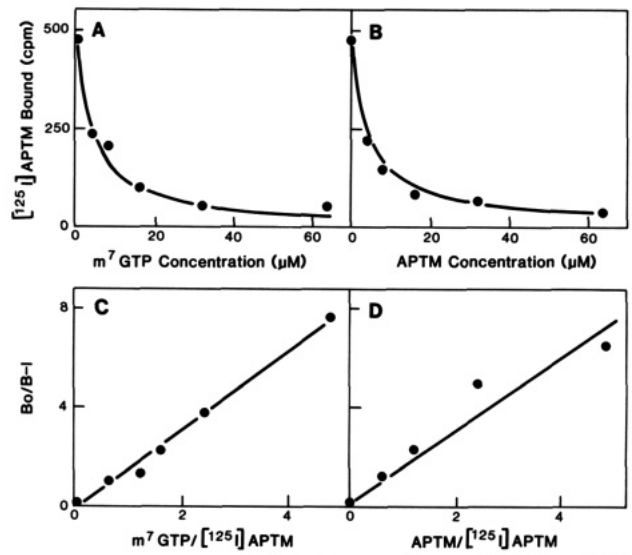

FIGURE 7: Competition of [^{125}I]APTM binding to eIF-4E by m^{7}GTP and APTM. The standard affinity labeling reaction was carried out as described under Materials and Methods by using 2 μM [^{125}I]APTM and varying amounts of either m^{7}GTP (A) or APTM (B) as competitor. Linear transforms (Ofengand and Henes, 1969) of the data in panels A and B are presented in panels C and D, respectively. From these, the ratio of dissociation constants for [^{125}I]APTM to that of competitor is calculated to be 1.6 in the case of m^{7}GTP and 1.5 in the case of APTM.

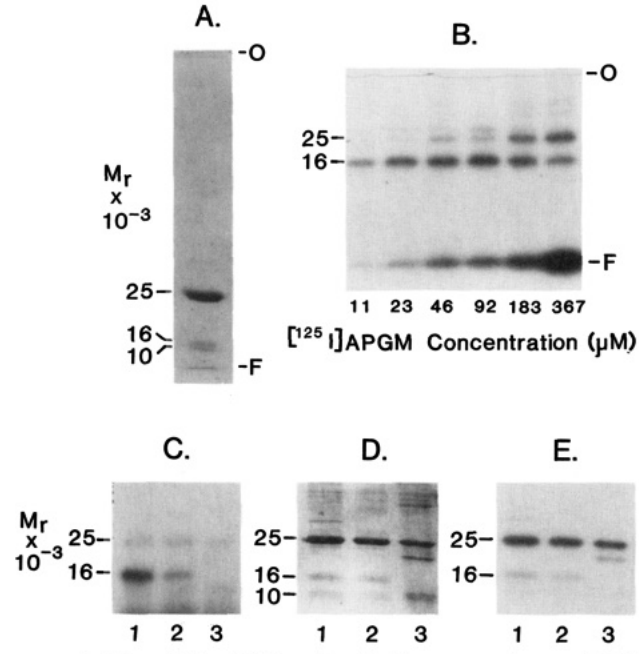

FIGURE 8: Photoaffinity labeling of purified human erythrocyte eIF-4E with [^{32}P]APGM. (A) Coomassie-stained SDS–PAGE (12.5% gel) of the eIF-4E preparation used in panel B, showing the 16-kDa degradation product (eIF-4E*) as well as a 10-kDa fragment. (B) Photoaffinity labeling of this eIF-4E preparation under the standard reaction conditions described in Methods using the indicated amounts of [^{125}I]APGM (1.4 Ci/mmol). SDS–PAGE was performed on a 17% gel and autoradiography was for 1 h. (C) Photoaffinity labeling of three different preparations of eIF-4E with [^{32}P]APGM (autoradiogram). (D) Coomassie-stained gel of the three preparations used in panel C. (E) Photoaffinity labeling of the same three preparations used in panels C and D with [^{32}P]BPM (autoradiogram).

(Figure 8B). Comparison of these results with those obtained with [^{125}I]APTM (Figure 6A) suggests that the cross-linking of [^{32}P]APGM to eIF-4E is reduced relative to m^{7}GTP. Half-maximal cross-linking to eIF-4E* occurred at approximately 20 μM (Figure 8B), comparable to the half-maximal cross-linking concentration of [^{125}I]APTM for eIF-4E of 20

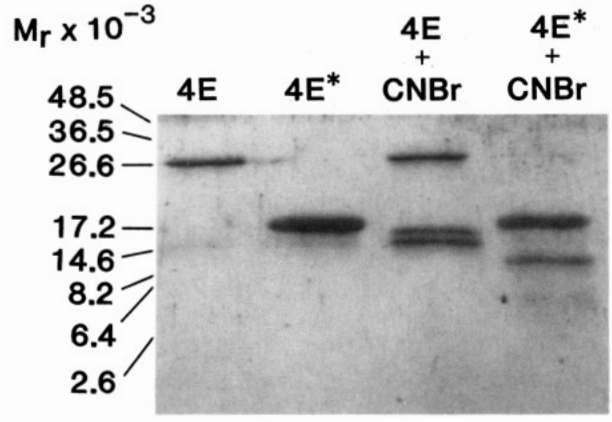

FIGURE 9: CNBr cleavage of eIF-4E and eIF-4E*. A human erythrocyte eIF-4E preparation was further purified by reverse-phase HPLC. Fractions containing eIF-4E (110 μg) and eIF-4E* (27 μg) were dried on a Speed-vac (Savant), dissolved in 50 μL of 70% formic acid (previously bubbled with N_2) and incubated with 50 nmol of Trp and 500 nmol of CNBr for 24 h at room temperature in the dark. Aliquots corresponding to 2.5 μg of undigested or CNBr-digested proteins were analyzed by SDS–PAGE on 20% gels together with Pharmacia low and high molecular weight standards. Positions and molecular weights of the standards are indicated.

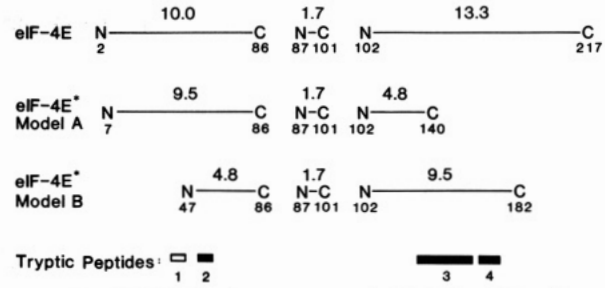

FIGURE 10: Models for the structure of eIF-4E*. CNBr-digested peptides of eIF-4E and the 16-kDa degradation product eIF-4E* are shown. The lengths of the lines are proportional to the sizes of the peptides, which are indicated above each line (in kilodaltons). The amino acids at the termini of each peptide are designated by numbers (below the lines), which refer to the primary sequence of eIF-4E (Rychlik et al., 1987a). Thus, cleavage by CNBr occurs on the C-terminal sides of Met-86 and Met-101. Two models for the structure of eIF-4E* are shown, based on the estimated sizes of the parent polypeptide (eIF-4E*) and the major CNBr fragment (9.5 kDa; Figure 9) as well as the primary structure of eIF-4E. Filled rectangles indicate the positions (in reference to the eIF-4E sequence) of tryptic peptides, which are common to both eIF-4E and eIF-4E*. The open rectangle indicates the position of a peptide found in eIF-4E but not in eIF-4E*. Identification of the peptides is given in the text.

μM (Figure 6A), whereas half-maximal labeling of eIF-4E with [^{32}P]APGM occurred at 150 μM (Figure 8B).

To test the possibility that photolysis of [^{32}P]APGM bound to eIF-4E actually caused hydrolysis of a peptide bond to *generate* eIF-4E*, we subjected three different preparations of eIF-4E that differed in their amounts of eIF-4E* (Figure 8D) to photolabeling with [^{32}P]APGM (Figure 8C) and [^{32}P]BPM (Figure 8E). [^{32}P]APGM labeled the most eIF-4E* in preparation 1, less in preparation 2, and none at all in preparation 3. If eIF-4E* had been generated by cleavage of a peptide bond during photolabeling, one would have expected the same amount of labeled eIF-4E* with all three preparations, since equal amounts of eIF-4E were used in all three reactions. Labeling the same three preparations with [^{32}P]-BPM was in proportion to the relative amounts of eIF-4E and eIF-4E* (Figure 8E).

Two approaches were taken to characterize eIF-4E* further. eIF-4E and eIF-4E* were reduced, carboxymethylated, and separated by reverse-phase HPLC. Analysis by 20% SDS–PAGE (Figure 9, lanes 4E and 4E*) revealed that the proteins were not cross-contaminated and allowed the molecular mass of eIF-4E* to be estimated as 16.0 kDa. The width of the eIF-4E* band suggested that it was somewhat heterogeneous in structure, presumably resulting from multiple sites of proteolytic cleavage at one or both termini. The HPLC-purified proteins were then cleaved with CNBr. The intact human eIF-4E contains two internal Met residues (Met-86 and Met-101; Rychlik et al., 1987a) from which is predicted three CNBr fragments of 10.0, 1.7, and 13.3 kDa. As shown in Figure 9, lane 4E + CNBr, two large fragments were observed in addition to uncleaved protein. Although these fragments migrated with mobilities corresponding to 15.8 and 14.7 kDa, on the basis of the protein standards, these are presumed to be the 13.3- and 10.0-kDa fragments, the discrepancy being attributable to the method of molecular mass estimation. The 1.7-kDa fragment is likely to have either run off the gel or not been stained sufficiently with Coomassie for detection.

When CNBr digestion products of eIF-4E* were examined, two bands were observed, the uncleaved starting material and a fragment estimated to be 9.5 kDa. From the molecular mass of the parent polypeptide (16 kDa), the 9.5-kDa digestion product, and the internal 1.7-kDa product, one can predict that a 4.8-kDa fragment should also be present. In fact, a faint band of the expected mobility appears in Figure 9 (lane 4E* + CNBr) below the 9.5-kDa fragment. It is not apparent why the recovery of this fragment would be less than that of the 9.5-kDa fragment, but one possibility is that the random proteolytic cleavage that produces eIF-4E* leaves a heterogeneous terminus. Regardless of the low yield of the 4.8-kDa fragment, it is clear that the CNBr fragments of eIF-4E* are different from those of eIF-4E. Thus, eIF-4E* is shorter at both N- and C-termini.

In order to map the 9.5-kDa fragment of eIF-4E* on the primary sequence of eIF-4E, tryptic peptides were generated from purified eIF-4E* and separated by HPLC. Selected peptides were identified by comparison of elution times with those of peptides derived from intact eIF-4E that had been sequenced (Rychlik et al., 1987a). Peptide HT15 represents amino acid residues 43–49 of eIF-4E and elutes at 24.5 min. It was not found in tryptic digests of eIF-4E* (designated peptide 1 in Figure 10). This suggests that Model A of Figure 10 is not correct, since peptide 1 would be present if the 9.5-kDa fragment were N-terminal. Peptides HT6 (residues 55-61; 14.5 min), HT23 (residues 129-157; 34 min), and HT12 (residues 163-173; 19.5 min) were all present in tryptic digests of eIF-4E* and are represented in Figure 10 as peptides 2, 3, and 4, respectively. Whereas the presence of peptide 2 supports either model, peptides 3 and 4 support only Model B. Thus, Model B is likely to represent the structure of eIF-4E*.

Discussion

The three probes employed in this study each have unique characteristics, advantages, and disadvantages. An advantage of [^{32}P]BPM is its reversible photoexcitation such that molecules that are not bound to protein at the time of initial irradiation can return to the ground state, bind to protein, and undergo further irradiation that leads to productive cross-linking. Also, the 320-nm excitation wavelength permits irradiation without interference by high protein or nucleotide concentrations, making it a favorable probe to use with whole-cell extracts or complex mixtures of initiation factor, nucleotides, and RNAs. Although measurement of the K_d of [^{32}P]BPM for eIF-4E was not performed in this study, it is clear from the data in Figure 4 that it labeled both eIF-4E

and eIF-4E* specifically in the nanomolar range. Probes utilizing [γ-^{32}P]GTP can be synthesized at extremely high specific activities. Although the procedure described under Materials and Methods typically yielded [^{32}P]GTP of 150 Ci/mmol, higher specific activities can be produced by adding less nonradioactive GTP at the end of [^{32}P]GTP synthesis; we have obtained preparations with specific activities as high as 430 Ci/mmol. The major disadvantages of [^{32}P]BPM are the time-consuming synthesis (1 week) using relatively large amounts of ^{32}P (20 mCi) and the acid lability of the P–N bond in the photoincorporated BPM–protein adducts.

The [^{32}P]APGM probe utilizes a different photoactive group than that of BPM and shares the advantage of high specific radioactivity with [^{32}P]BPM. However, it also shares the disadvantages of a time-consuming synthesis with ^{32}P-labeled intermediates and lability of the P–N bond. An unexpected property was its ability to label eIF-4E* preferentially over eIF-4E.

The [^{125}I]APTM probe has two major advantages over the others. The first is the ease of producing the final radiolabeled compound. The nonradioactive precursor **11** is stable and can be conveniently synthesized in moderately large quantities (e.g., 7 μmol) and then quantitatively radioiodinated in small quantities (e.g., 50 nmol) as needed. Although [^{125}I]APTM with specific activities of 2–5 Ci/mmol were routinely produced, this only reflected the amount of carrier-free Na^{125}I used in the reaction. In some instances, specific activities as high as 50 Ci/mmol were obtained, but even this is still far below the theoretical maximum. The second major advantage of [^{125}I]APTM is the location of the radioactive label relative to the sensitive P–N bond. Cleavage of this P–N bond in the covalent photo-cross-linked adduct would leave the labeled tyrosine moiety attached to the protein. This feature would aid in the identification of active-site peptides because acid conditions could be employed for chromatography without fear of losing the crucial radiolabel. Furthermore, after removal of the m^{7}GTP moiety, the size and charge of such derivatized peptides would be more similar to those of the respective parent peptides than with [^{32}P]APGM and [^{32}P]BPM, facilitating identification on reverse-phase HPLC. A theoretical disadvantage of APTM is the steric bulk of the photoactive group and spacer arm. However, the saturation experiment yielded a K_d of 6.9 μM for ^{125}I-ATPM (Figure 6), which compares favorably with that of m^{7}GTP (Carberry et al., 1989), and the competition experiment (Figure 7) demonstrated that m^{7}GTP and APTM were indistinguishable as competitors. Consequently, it may be concluded that, within the limits of the experimental technique, APTM and m^{7}GTP bind to eIF-4E with the same affinity.

The 16-kDa species designated eIF-4E* has been reported previously as a contaminant of eIF-4E preparations (Rychlik et al., 1986; Buckley & Ehrenfeld, 1986). The present study confirms that this species contains a cap-binding site, as it was labeled with [^{32}P]BPM (Figures 4 and 8E), [^{125}I]APTM (Figure 5), and [^{32}P]APGM (Figure 8B,C). The contention that it represents a proteolytic breakdown product of eIF-4E is based on this and the presence of many common tryptic peptides. These peptides could be used to infer further details of the eIF-4E* structure. The data summarized in Figure 10 indicate that eIF-4E* begins around residue 47 and ends at around residue 182. Such a fragment would contain the major phosphorylation site, Ser-53 (Rychlik et al., 1987b). This is supported by the observations that two forms of eIF-4E* are detected by isoelectric focusing (Rychlik et al., 1986) and that extracts of cultured lymphocytes labeled with ^{32}P yield labeled forms of both eIF-4E and eIF-4E* when passed over m^{7}GTP-Sepharose (W. Rychlik and R. Rhoads, unpublished work).

With two of the probes ([^{32}P]BPM and [^{125}I]APTM), there appeared to be no difference in affinity between eIF-4E and eIF-4E*, from which we conclude that the truncated form of eIF-4E, representing only 63% of the amino acid residues, retains the entire cap-binding site. The fact that the third probe, [^{32}P]APGM, cross-linked normally to eIF-4E* but poorly to eIF-4E is more difficult to interpret. Conceivably APGM adopts a conformation, not available to BPM or APTM, that fits well in the cap-binding site of eIF-4E* but poorly into that of eIF-4E. This interpretation in turn raises the intriguing possibility that a domain of eIF-4E that is missing in eIF-4E* partially occludes the cap-binding site, perhaps for purposes of regulation or specificity of binding.

Acknowledgments

We thank Dr. A. Jeganathan for developing part of the route shown in Figure 2, Dr. Erik Patzelt and Eva Thalmann for help with [^{32}P]BPM synthesis, and Dr. Boyd Haley for helpful discussions.

Registry No. **1**, 83454-50-6; **1** (unlabeled), 80904-71-8; **2**, 126848-03-1; **3**, 126823-92-5; **3** (diiodo derivative), 126848-05-3; **4**, 100-01-6; **5**, 83209-85-2; **6**, 126823-99-2; **6**·HCl, 105310-99-4; **7**, 126823-93-6; **8**, 106-50-3; **9**, 126823-94-7; **10**, 126824-02-0; **10**·HCl, 126823-95-8; **11**, 126848-04-2; **12a**, 126823-96-9; **12b**, 126823-98-1; **13**, 126823-97-0; m^{7}GTP·2Na, 104809-18-9; [γ-^{32}P]mGTP, 126824-00-8; *N*-(4-nitrophenyl)-α-(*tert*-butoxycarbamoyl)acetamide, 82518-64-7; *N*-(*tert*-butoxycarbamoyl)glycine, 4530-20-5; *N*-(4-azidophenyl)-α-(*tert*-butoxycarbamoyl)acetamide, 105310-98-3; *N*-(*tert*-butoxycarbonyl)tyrosine, 3978-80-1; *N*-(4-aminophenyl)-2-(*tert*-butoxycarbamoyl)-3-(4-hydroxyphenyl)propionamide, 126824-01-9; *N*-(4-azidophenyl)-2-amino-3-(4-hydroxy-3-iodophenyl)propionamide, 126824-03-1.

References

Allen, G. (1981) in *Laboratory Techniques in Biochemistry and Molecular Biology* (Work, T. S., Burdon, R. H., Eds.) Vol. 9, Sequencing of Proteins and Peptides, p 62, Elsevier/North Holland, Amsterdam.

Babkina, G. T., Zarytova, V. F., & Knorre, D. G. (1975) *Bioorg. Khim. 1*, 611.

Babkina, G. T., Jonak, J., & Rychlik, I. (1982) *Biochim. Biophys. Acta 698*, 116.

Bayley, H., & Knowles, J. R. (1977) *Methods Enzymol. 46*, 69.

Blaas, D., Patzelt, E., & Kuechler, E. (1982) *Nucleic Acids Res. 10*, 4803.

Bradford, M. M. (1976) *Anal. Biochem. 72*, 248.

Buckley, B., & Ehrenfeld, E. (1986) *Virology 152*, 497.

Carberry, S. E., Rhoads, R. E., & Goss, D. J. (1989) *Biochemistry 28*, 8078.

Carberry, S. E., Darzynkiewicz, E., Stepinski, J., Tahara, S. M., Rhoads, R. E., & Goss, D. J. (1990) *Biochemistry 29*, 3337.

Edery, I., Humbelin, M., Darveau, A., Lee, K. A. W., Milburn, S., Hershey, J. W. B., Trachsel, H., & Sonenberg, N. (1983) *J. Biol. Chem. 258*, 11398.

Freifelder, D. M. (1982) *Physical Biochemistry*, p 654, W. H. Freeman and Company, San Francisco.

Glonek, T., Kleps, R. A., & Myers, T. C. (1974) *Science 185*, 352.

Glynn, I. M., & Chappell, J. B. (1964) *Biochem. J. 90*, 147.

Grifo, J. A., Tahara, S. M., Morgan, M. A., Shatkin, A. J., & Merrick, W. C. (1983) *J. Biol. Chem. 258*, 5804.

Hellmann, G. M., Chu, L.-Y., & Rhoads, R. E. (1982) *J. Biol. Chem. 257*, 4056.

Hiremath, L. S., Hiremath, S. T., Rychlik, W., Joshi, S., Domier, L. L., & Rhoads, R. E. (1989) *J. Biol. Chem. 264*, 1132.
Johnson, R. A., & Walseth, T. F. (1979) in *Advances in Cyclic Nucleotide Research* (Brooker, G., Greengard, P., & Robison, G. A., Eds.) Vol. 10, p 135, Raven Press, New York.
Knorre, D. G., Kurbatov, V. A., & Samukov, V. V. (1976) *FEBS Lett. 70*, 105.
Kometani, T., Watt, D. S., Ji, T., & Fitz, T. (1985) *J. Org. Chem. 50*, 5384.
Laemmli, U. K., & Favre, M. (1973) *J. Mol. Biol. 80*, 575.
Levya, E., Platz, M. S., Persey, G., & Wirz, J. (1986) *J. Am. Chem. Soc. 108*, 3783.
Markwell, M. A. K., & Fox, C. F. (1978) *Biochemistry 17*, 4807.
McCubbin, W. D., Edery, I., Altmann, M., Sonenberg, N., & Kay, C. M. (1988) *J. Biol. Chem. 263*, 17663.
McCubbin, W. D., Edery, I., Altmann, M., Sonenberg, N., & Kay, C. M. (1989) *FEBS Lett. 245*, 261.
Ofengand, J., & Henes, C. (1969) *J. Biol. Chem. 244*, 6241.
Patzelt, E., Blaas, D., & Kuechler, E. (1983) *Nucleic Acids Res. 11*, 5821.
Pelletier, J., & Sonenberg, N. (1985) *Mol. Cell. Biol. 5*, 3222.
Ray, B. K., Lawson, T. G., Kramer, J. C., Cladaras, M. H., Grifo, J. A., Abramson, R. D., Merrick, W. C., & Thach, R. E. (1985) *J. Biol. Chem. 260*, 7651.
Rhoads, R. E. (1988) *Trends Biochem. Sci. 13*, 52.
Rychlik, W., Gardner, P. R., Vanaman, T. C., & Rhoads, R. E. (1986) *J. Biol. Chem. 261*, 71.
Rychlik, W., Domier, L. L., Gardner, P. R., Hellmann, G. M., & Rhoads, R. E. (1987a) *Proc. Natl. Acad. Sci. U.S.A. 84*, 945.
Rychlik, W., Russ, M. A., & Rhoads, R. E. (1987b) *J. Biol. Chem. 262*, 10434.
Shields, C. J., Chrisope, D. R., Schuster, G. B., Dixon, A. J., Poliakoff, M., & Turner, J. J. (1987) *J. Am. Chem. Soc. 109*, 4723.
Smith, P. A. S., Hall, J. H., & Kan, R. O. (1962) *J. Am. Chem. Soc. 84*, 485.
Sonenberg, N. (1981) *Nucleic Acids Res. 9*, 1643.
Sonenberg, N. (1988) *Prog. Nucleic Acid Res. Mol. Biol. 35*, 173.
Sonenberg, N., & Shatkin, A. J. (1977) *Proc. Natl. Acad. Sci. U.S.A. 74*, 4288.
Sonenberg, N., Rupprecht, K. M., Hecht, S. M., & Shatkin, A. J. (1979) *Proc. Natl. Acad. Sci. U.S.A. 76*, 4345.
Tahara, S. M., Morgan, M. A., & Shatkin, A. J. (1981) *J. Biol. Chem. 256*, 7691.
Webb, N. R., Chari, R. V. J., DePillis, G., Kozarich, J. W., & Rhoads, R. E. (1984) *Biochemistry 23*, 177.

Conformation of Methyl β-Lactoside Bound to the Ricin B-Chain: Interpretation of Transferred Nuclear Overhauser Effects Facilitated by Spin Simulation and Selective Deuteration†

V. L. Bevilacqua, D. S. Thomson,‡ and J. H. Prestegard*
Department of Chemistry, Yale University, New Haven, Connecticut 06511
Received November 28, 1989; Revised Manuscript Received February 16, 1990

ABSTRACT: Spin simulation and selective deuteration have been used to aid in the interpretation of 1D transferred nuclear Overhauser effect (TRNOE) NMR experiments on ricin B-chain/ligand systems. Application of these methods has revealed a change in the conformation of deuterated methyl β-lactoside upon binding to the ricin B-chain which results in a slight change in glycosidic torsional angles which appear to dominate in the solution conformation. The combination of simulation and experiment also shows an important sensitivity of TRNOE magnitudes to dissociation rate constants and available spin-diffusion pathways for the ricin B-chain/ligand systems under study. The sensitivity to dissociation rates allows determination of rate constants for methyl β-lactoside and methyl β-galactoside of 50 and 300 s^{-1}, respectively.

Cell membranes must interact with their external environment as a part of important biological processes such as cell recognition, intercellular adhesion, and growth regulation. Moreover, a number of pathological events begin with a recognition step at the cell membrane. Specific peptides and proteins, including hormones, antibodies, viral coat proteins, bacterial toxins, and lectins, frequently mediate interactions with the cell. Thus, one step toward understanding the interactions of a cell with its environment begins with understanding these mediator molecules and their interaction with membranes.

Ricin, a toxic lectin of MW 65 000 found in castor bean seeds, shares a general subunit composition and membrane interaction mode with several other toxic lectins and agglutinins (Olsnes & Pihl, 1982). Especially notable is its relation to *Ricinus communis* agglutinin, a larger lectin known to mediate intermembrane contact and thereby specifically enhance Ca^{2+}-induced fusion of certain membranes (Sundler & Wijkander, 1983). Ricin contains two subunits, the A- and B-chains (Ra and Rb,[1] respectively), covalently linked by a disulfide bond. The A-chain (MW 31 000) inhibits protein synthesis by inactivating the 60S ribosomal subunit. The

† This work was supported by U.S. Public Health Service Grant GM19035.
* Author to whom correspondence should be addressed.
‡ Present address: Cytel Corp., 11094 N. Torrey Pines Rd., La Jolla, CA 92037.

[1] Abbreviations: NOE, nuclear Overhauser effect; Rb, ricin B-chain; FID, free induction decay; 2D NOESY, two-dimensional NOE spectroscopy; SIR, selective inversion recovery; ISPA, independent spin pair approximation.

0006-2960/90/0429-5529$02.50/0