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Partial Purification and Characterization of Two Hen Oviduct Protein Synthesis Initiation Factors Capable of Initiation Complex Formation†

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ABSTRACT: Two initiation factors aIF-2A and aIF-2 capable of binding Met-tRNA$_f$ to form an initiation complex in vitro have been isolated from the hen oviduct. Both factors were obtained from a 0.5 M KCl wash of a nuclear-microsomal fraction of the oviduct homogenate. Four purification steps were carried out according to the protocol for the purification of rabbit reticulocyte eIF-2A before a high molecular weight ribonuclease was removed by molecular sieve chromatography and aIF-2A activity was detected. This oviduct factor has been characterized with respect to its activity in stimulating poly(U)-directed polyphenylalanine synthesis, lowering the Mg^{2+} concentration optimum required for polyphenylalanine synthesis, and stimulating phenylalanyl-puromycin synthesis. In these assays aIF-2A was able to substitute for the corresponding rabbit reticulocyte factor. Factor aIF-2A was capable of binding fMet-tRNA$_f$ and Met-tRNA$_f$ to 40S ribosomal subunits programmed with the AUG codon. The molecular weight and Stokes radius of aIF-2A were estimated by molecular sieve chromatography to be 110 000 and 42.5 Å, respectively. The sedimentation coefficient was 3.5 S as measured by sedimentation analysis on sucrose density gradients. Combining these data, the calculated molecular weight of aIF-2A was 62 500. These values are similar to those reported for the highly asymmetric reticulocyte factor. The activity of the second oviduct factor aIF-2 was detected during gradient elution chromatography of the nuclear-microsomal salt wash on DEAE-cellulose. This factor bound Met-tRNA$_f$ in a binary complex since GTP was not required and did not further increase the binding. This may be caused by the aging of the factor and/or its partial degradation. Similar characteristics have been reported for the corresponding reticulocyte factor, eIF-2. Nevertheless, aIF-2 was capable of forming an 80S initiation complex as determined by the stimulation of methionyl-puromycin synthesis upon the addition of ribosomes and ribosomal subunit joining factors, eIF-5 and eIF-4C and D. The molecular weight of aIF-2 was estimated by molecular sieve chromatography to be 102 000 corresponding to the value reported for the monomer form of the reticulocyte factor.

There is evidence that sex steroid hormones increase the rate of protein synthesis (Palmiter et al., 1970, 1971; Palmiter, 1972a,b; Comstock et al., 1972) as well as increasing the cellular concentration of translatable mRNA in the oviduct (Rosen & O'Malley, 1975; Schimke et al., 1975; Harris et al., 1975). We are interested in learning the mechanisms by which protein synthesis is regulated at the translation level in this tissue and whether hormones act directly or indirectly in this control. We have chosen to use for these investigations the estrogen-stimulated chicken oviduct, a widely used model system for studying the actions of sex steroid hormones. Although both initiation and elongation factors have been well studied in several mammalian and eukaryotic systems, it is necessary systematically to isolate and characterize these factors in the oviduct in order to study the molecular events by which hormones affect protein synthesis.

Recently our laboratory reported on the properties of two protein synthesis initiation factors designated A2A and A2B

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(aIF-5 and aIF-4C and D,[1] respectively) from the hen oviduct (Hejtmancik & Comstock, 1976). This was the first description of these ribosomal joining factors isolated from an avian system. In this communication we describe the preparation and characteristics of two additional hen oviduct factors designated aIF-2A and aIF-2 which bind the initiator tRNA, Met-tRNA$_f$, into an initiation complex.

Two distinct protein factors are capable of binding Met-tRNA$_f$ to form an initiation complex during in vitro eukaryotic protein synthesis. One of these factors, designated eIF-2A, was isolated from the 0.5 M KCl wash of rabbit reticulocyte polysomes. The factor was also required for the poly(U)-directed synthesis of polyphenylalanine at low Mg^{2+} concentrations (Shafritz & Anderson, 1970). It has been shown that crude eIF-2A was also required for the synthesis of globin from endogenous mRNA (Prichard et al., 1970). eIF-2A-like factors have been isolated subsequently in other laboratories from supernatant fractions as well as the high salt washes of polysomes from a variety of tissues. They were capable of catalyzing the binding of fMet-tRNA$_f$, Phe-tRNA, or acPhe-tRNA in the presence of the appropriate codons to salt washed ribosomes or 40S subunits (Zasloff & Ochoa, 1971; Gasior & Moldave, 1972; Leader & Wool, 1972; Picciano et al., 1973; Eich & Drews, 1974; Cimadevilla & Hardesty, 1975; Grummt, 1974; Berthelot et al., 1973). GTP was, however, not required for these binding reactions (Zasloff & Ochoa, 1972; Nombela et al., 1975; Merrick & Anderson, 1975). eIF-2A was, nevertheless, capable of binding the natural unformylated initiator tRNA to the AUG codon (Merrick & Anderson, 1975). The initiation factors eIF-5 and eIF-4C stimulated this reaction and GTP was then required (Shafritz et al., 1972). More recently, however, using a homogeneous preparation of eIF-2A the requirements for eIF-5 and GTP were lost while stimulation by eIF-4C was retained (Adams et al., 1975).

The other factor designated eIF-2 formed a ternary or binary complex with Met-tRNA$_f$ and/or GTP. These complexes were formed in the absence of ribosomes and templates and bound to nitrocellulose filters (Levin et al., 1973; Schreier & Staehelin, 1973; Gupta et al., 1973; Cashion & Stanley, 1974; Filipowicz et al., 1975; Smith & Henshaw, 1975; Tredwell & Robinson, 1975). Present data indicated that both of these factors formed functional initiation complexes, although the efficiency of eIF-2 was greater in forming the initial dipeptide, methionyl-puromycin, in the presence of endogenous mRNA (Adams et al., 1975). Moreover, the ternary complex (eIF-2·Met-tRNA$_f$·GTP) was highly stable while the quaternary complex (eIF-2A·40S subunit·Met-tRNA$_f$·AUG) was unstable (Zasloff & Ochoa, 1973). At the present time the exact role in vivo of these two factors is not understood since each one is capable of forming in vitro an 80S initiation ribosome.

Experimental Procedures

All procedures involving materials with biological activity were carried out at 2–4 °C.

Materials

Chromatography materials were obtained from the following suppliers: DEAE-cellulose (DE-23) and phosphocellulose (P11) (Whatman) and Sephadex G and CM type gels (Pharmacia). Radiochemicals were purchased from Amersham/Searle: [^{14}C]Phenylalanine (522 mCi/mmol) and [^{3}H]methionine (7.7 Ci/mmol). Poly(U), AUG, and *Escherichia coli* K-120 tRNA were obtained from Miles Laboratories. Rat liver tRNA^{fMet} was obtained from Biogenics Research Corp. The cell paste of *Escherichia coli* was purchased from General Biochemical Corp. Dithiothreitol, ATP, GTP, phosphoenolpyruvate, and pyruvate kinase were purchased from Calbiochem and puromycin dihydrochloride was from Nutritional Biochemical Corp. Catalase, aldolase, bovine albumin, ovalbumin, chymotrypsinogen A, myoglobin, cytochrome *c*, and monothioglycerol were purchased from Sigma Chemical Co. Sparsomycin was obtained from the National Cancer Institute, National Institutes of Health. All other materials used were of reagent grade quality. Ultrafiltration equipment and membranes were obtained from Amicon Corp. Frozen hen oviducts were supplied by Pel-Freeze Biologicals.

Methods

Preparation of Rabbit Reticulocyte Salt-Washed Ribosomes and Initiation Factors. Rabbit reticulocyte lysate was prepared and the 0.5 M KCl wash of polysomes and salt washed ribosomes were isolated. Reticulocyte initiation factors were separated from the salt wash and purified as described (Merrick et al., 1974; Prichard & Anderson, 1974).

Reticulocyte Supernatant Fraction. The high speed supernatant was prepared from reticulocyte lysate by the method of Moldave et al. (1971). Partially purified elongation factors (EF-1 and EF-2) were isolated from this supernatant as described previously (Hejtmancik & Comstock, 1976).

Preparation of [^{14}C]Phe-tRNA. This material was prepared as previously described by Anderson (1969) using synthetases prepared by the method of Muench & Berg (1966). The [^{14}C]Phe-tRNA had a specific activity of 1040 cpm/pmol. The unfractionated tRNA was 1.3% acylated and 90% of the total radioactivity was precipitable in cold trichloroacetic acid.

Preparation of [^{3}H]Met-tRNA$_f$. Methods similar to those described by Shafritz & Anderson (1970) were used for the acylation of methionyl-tRNA$_f$. The formylated species was prepared by the addition of 0.6 mg of calcium leucovorin to the reaction. The incubation contained 4.0 A_{260} units of partially purified rabbit liver tRNA^{fMet}, 52 nmol of [^{3}H]methionine, and saturating amounts of *Escherichia coli* aminoacyl-tRNA synthetase (Muench & Berg, 1966). Of the added tRNA, 4.4% was aminoacylated and 43% of the radioactivity was precipitable in cold trichloroacetic acid. The acid precipitated material was determined to be completely formylated (Leder & Bursztyn, 1966). The specific activity of the [^{3}H]Met-tRNA$_f$ was 4930 cpm/pmol.

Isolation of Reticulocyte 40S Ribosomal Subunits. Active 40S ribosomal subunits were prepared by incubation with puromycin and 10–30% sucrose gradient centrifugation as previously described (Hejtmancik & Comstock, 1976).

Poly(U)-Directed Polyphenylalanine Synthesis from [^{14}C]Phe-tRNA, Poly(U) Assay. eIF-2A activity was routinely tested using the poly(U) assay at 5 mM $MgCl_2$ concentration (unless otherwise noted) as described previously (Hejtmancik & Comstock, 1976). Each of the complementary reticulocyte factors required for this assay were used in saturating amounts (eIF-5, eIF-4C and D, EF-1 and EF-2).

Aminoacyl-puromycin Dipeptide Synthesis. Synthesis of the dipeptide aminoacyl-puromycin was carried out by the

[1] In this communication initiation factors will be designated according to the newly suggested nomenclature as outlined by Anderson et al., 1977: eIF (eukaryotic initiation factor), the e will be replaced by a for avian, followed by the designated factor number. The NIH factor M1 will be designated eIF-2A. Abbreviations used: DEAE, diethylaminoethyl; CM, carboxymethyl; AUG, adenylyl-3′,5′-uridyl-3′,5′-guanosine 3′-phosphate; poly(U), poly(uridylic acid); Na_2EDTA, disodium ethylenediaminetetraacetate; Tris, tris(hydroxymethyl)aminomethane; EF, elongation factor; Hepes, *N*-2-hydroxyethylpiperazine-*N*′-2-ethanesulfonic acid.

method of Shafritz et al. (1970). Fifty-microliter reaction mixtures were incubated at 30 °C for 10 min and contained: 20 mM Tris-HCl (pH 7.2 at 25 °C), 150 mM KCl, 2 mM $MgCl_2$, 0.5 mM GTP, 1 mM dithiothreitol, 1.0 A_{260} unit of salt-washed reticulocyte ribosomes, 0.5 mM puromycin (neutralized to pH 7.0 with 0.1 N KOH), 0.5 A_{260} unit of poly(U) or 0.25 A_{260} unit of AUG, 10 pmol of [^{14}C]Phe-tRNA or 7.5 pmol of [^{3}H]Met-tRNA$_f$, and complementary reticulocyte factors as indicated in the figures. The peptidyl-puromycin was extracted into ethyl acetate by the method of Leder & Bursztyn (1966). The radioactivity in the extract was counted in Spectrofluortoluene–Triton X-100 (2:1 v/v) at an efficiency of 75% for carbon-14.

[^{3}H]fMet-tRNA$_f$ Binding to Reticulocyte 40S Ribosomal Subunits. The AUG-directed binding of [^{3}H]fMet-tRNA$_f$ to 40S ribosomal subunits was performed as described previously in detail (Hejtmancik & Comstock, 1976). Dried filters were counted in spectrofluor-toluene with an efficiency of 33%.

[^{3}H]Met-tRNA$_f$·GTP·eIF-2 Complex Formation. The assay was performed according to Safer et al. (1975b). Reactions were initiated by the addition of factors to the mixture and incubation was carried out for 2 min at 0 °C. The reaction was terminated by dilution with buffer: 50 mm kcl, 20 mM Tris-HCl (pH 7.3 at 4 °C), 10 mM $MgCl_2$, and 1 mM D,L-methionine. Complexes were collected on nitrocellulose filters and washed with the same buffer. Filters were dried and counted as described above.

Preparation of Hen Oviduct Initiation Factor aIF-2A. Preparation of the nuclear microsomal salt wash fraction has been previously described (Hejtmancik & Comstock, 1976). The crude extract from 400 g of frozen oviduct served as the starting material for the isolation of aIF-2A. Salt wash of 650 mL (19 500 mg of protein) was dialyzed against 4 × 6 L of buffer [C 20 mM Tris-HCl (pH 7.5 at 4 °C), 10 mM $MgCl_2$, 0.1 mM Na_2EDTA (pH 7.0), 0.1% (v/v) monothioglycerol] made 100 mM in KCl. The dialyzed material was applied to a DEAE-cellulose (DE-23) column (2.6 × 40 cm) equilibrated with buffer C made 100 mM in KCl. The nonabsorbed material was collected (700 mL, 15 400 mg of protein) and fractionated (40–60% saturation) by adding powdered $(NH_4)_2SO_4$ containing 2% (w/w) $(NH_4)_2CO_3$. The sample (84 mL, 1600 mg of protein) was initially dialyzed against buffer C made 200 mM in KCl and then titrated to pH 7.9 by the addition of 0.5 M Tris-HCl (pH 7.9 at 4 °C). The sample was applied at a rate of 35 mL/h to a phosphocellulose (P-11) column (1.6 × 30 cm) equilibrated with buffer C (minus $MgCl_2$) made 200 mM in KCl. The column was washed with this buffer until A_{280} absorbing material was no longer eluted. The salt concentration was increased to 350 mM KCl and the column again washed until A_{280} absorbing material was eluted. The protein eluting between 350 and 550 mM KCl was concentrated by ultrafiltration against a UM-2 membrane (2 mL, 10 mg of protein). The sample was applied to a Sephadex G-200 column (1.6 × 90 cm) equilibrated with buffer C made 200 mM in KCl. The column was eluted with the same buffer at a flow rate of 10 mL/h and 4-mL fractions were collected. Fractions showing aIF-2A activity were pooled and concentrated by ultrafiltration against a UM-2 membrane (4 mL, 0.8 mg of protein). The samples were stored in liquid nitrogen.

Hen oviduct aIF-2A was also isolated with the following modified procedure. A 40–70% ammonium sulfate fractionation of the nuclear microsomal salt wash fraction was performed rather than the 0–90% ammonium sulfate concentration step. This fraction (100 mL, 2300 mg of protein) was resuspended and dialyzed against buffer S (10 mM Hepes, pH 6.2, 1 mM $MgCl_2$, 0.1 mM Na_2EDTA, pH 7.0, 0.1% monothioglycerol, and 50 mM KCl). The sample was applied to a CM-Sephadex (CM-50) column (2.6 × 22 cm) equilibrated with the same buffer. The column was washed until all the nonabsorbing material had been eluted. A linear gradient, 50 to 250 mM KCl in buffer S (200 × 200 mL), was used to elute the aIF-2A activity. The column flow rate was 20 mL/h and 5-ml fractions were collected. Fractions containing aIF-2A activity were pooled, concentrated by ultrafiltration against a UM-2 membrane (5 mL, 40 mg of protein), and stored in liquid nitrogen.

Preparation of Hen Oviduct Initiation Factor aIF-2. The nuclear microsomal salt wash fraction from 236 g of hen oviduct was absorbed to the DEAE-cellulose column and eluted as previously described (Hejtmancik & Comstock, 1976). Fractions containing Met-tRNA$_f$ binding activity were pooled, concentrated by ammonium sulfate fractionation (40–60%), and stored in liquid nitrogen. The sample (7 mL, 60 mg of protein) was dialyzed against buffer C made 200 mM in KCl and applied to an Ultrogel ACA-34 column (1.6 × 90 cm) equilibrated in the same buffer. The flow rate was 8 mL/h and 3-mL fractions were collected. Fractions with binding activity were pooled, concentrated by ultrafiltration with a UM-2 membrane, and stored in liquid nitrogen. The sample was titrated to pH 7.9 as described above and applied to a phosphocellulose (P-11) column (1.6 × 10 cm) equilibrated with buffer C (minus $MgCl_2$) made 200 mM in KCl. The column was washed with this initial buffer and then the salt concentration was increased to 400 mM. After no further A_{280} absorbing material was eluted, the salt concentration was increased to 800 mM. The fractions containing protein were concentrated by ultrafiltration and stored in small aliquots in liquid nitrogen.

Determination of Apparent Molecular Weight and Stokes Radius by Molecular Sieve Chromatography. Two milliliters of aIF-2A (1.3 mg/mL) isolated by G-200 chromatography were applied to 1.6 × 90 cm analytical column of Sephadex G-200. The column was equilibrated and eluted with buffer C made 200 mM in KCl at a flow rate of 4 mL/h. Two-milliliter fractions were collected. The void volume and total column volume were determined with the University Dye Kit (Pharmacia). Highly purified proteins of known molecular weights were used as standards: catalase (bovine liver) 220 000; aldolase (rabbit muscle), 158 000; collagenase (*Cl. histolyticum*), 102 000; bovine albumin, 67 000; ovalbumin, 46 000; chymotrypsinogen A (bovine pancreas), 25 000; myoglobin (whale skeletal muscle), 17 500; and cytochrome *c* (horse heart), 12 300. Elution volumes were determined by absorbance at A_{280} with a UA-5 monitor (ISCO). Aliquots of 25 μL were analyzed for aIF-2A activity with the poly(U) assay. Values for Stokes radii of the standards were obtained from the data of Siegel & Monty (1966). The Stokes radius of aIF-2A was determined as described by Laurent & Killander (1964).

Determination of Sedimentation Coefficient. aIF-2A (200 μL, 40 μg of protein) was layered on 5-mL linear 5–20% sucrose gradients (containing buffer C made 200 mM in KCl). The following protein standards with known sedimentation coefficients were used: aldolase, 8.8 S; bovine albumin, 4.27 S; ovalbumin, 3.54 S; and chymotrypsinogen A, 2.58 S (Smith, 1970). The samples were centrifuged in a Beckman SW 50.1 rotor for 16 h at 189 000*g*. Fractions (1.3 mL) were collected with a Model 640 density gradient fractionator (ISCO). Elution volumes were determined by absorbancy at A_{280} with a UA-5 monitor (ISCO). Aliquots of 20 μL were analyzed for aIF-2A activity with the poly(U) assay.

Measurement of Protein and Nucleic Acid Content. These

analyses were performed as described previously (Hejtmancik & Comstock, 1976). Protein was assayed by a modification of the method of Lowry et al. (1951). Nucleic acid content was estimated by measuring the absorbance at 260 nm.

Results

Isolation of Hen Oviduct aIF-2A. The methods employed for the partial purification of aIF-2A are given under Experimental Procedures. As reported previously, the 0.5 M KCl salt wash of the hen oviduct nuclear microsomal fraction completely inhibited protein synthesis (Hejtmancik & Comstock, 1976). The fraction of salt wash absorbed to DEAE-cellulose at 100 mM KCl was, however, not inhibitory when eluted from the column by gradient elution chromatography. The column flow through fraction, however, inhibited protein synthesis. It was from this fraction that the initiation factor eIF-2A which bound the initiator tRNA to the 40S ribosomal subunit had been isolated from rabbit reticulocyte and liver polysomal high salt washes. There was the possibility that an aIF-2A activity was present in the hen oviduct but was masked by the presence of inhibitors which coeluted with the factor during DEAE-cellulose chromatography. To test for this possibility the nonabsorbed fraction was purified further by the additional steps described for the rabbit reticulocyte and liver (Merrick & Anderson, 1975; Picciano et al., 1973). The first three steps (ammonium sulfate fractionation, phosphocellulose chromatography, and molecular sieve chromatography) in this purification scheme were carried out before aIF-2A activity was detected by the poly(U)-directed synthesis of polyphenylalanine from [^{14}C]Phe-tRNA at low magnesium concentration.

A peak of activity was observed for the first time during molecular sieve chromatography. The activity was followed immediately by an inhibitor(s) which depressed the background incorporation of [^{14}C]Phe-tRNA by more than 50%. The nature of the inhibitor was investigated in several preliminary experiments and identified as a RNase of apparent high molecular weight. Since RNase is tightly absorbed during carboxymethylcellulose chromatography at slightly acid pH, a possible method for the purification of aIF-2A was cationic exchange chromatography. Therefore, the 40–60% ammonium sulfate fractionation of the nuclear microsomal salt wash was absorbed to a Sephadex CM-50 column and eluted by a linear salt gradient. aIF-2A activity was eluted at 140 mM KCl and separated from the bulk of the protein applied to the column (data not shown). Unfortunately, aIF-2A isolated by this technique was highly unstable and the method was consequently abandoned. Nevertheless, the ion-exchange behavior of the oviduct activity was in close agreement with that described for reticulocyte eIF-2A (Merrick & Anderson, 1975).

Biological Properties of aIF-2A. Hen oviduct aIF-2A prepared by Sephadex G-200 chromatography was tested for activity in several in vitro protein synthesis initiation reactions and subreactions. Oviduct aIF-2A stimulated the poly(U)-directed polymerization of polyphenylalanine from [^{14}C]-Phe-tRNA at 5 mM Mg^{2+} concentration in a manner similar to reticulocyte eIF-2A (Figure 1A). The system which was rate limiting in salt-washed ribosomes demonstrated a linear increase in activity upon the addition of factor. Oviduct aIF-2A and reticulocyte eIF-2A were completely interchangeable stimulating the incorporation equally when added to the assay in saturating amounts.

The Mg^{2+} concentration optimum for the poly(U) assay was found to be 5–6 mM in the presence of saturating amounts of oviduct initiation factor aIF-2A (Figure 1B). In the presence

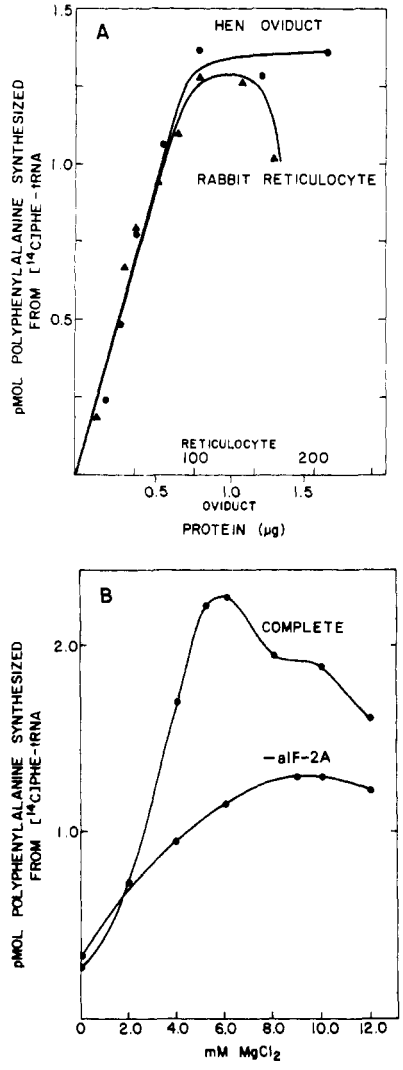

FIGURE 1: The effect of increasing amounts of hen oviduct aIF-2A and rabbit reticulocyte eIF-2A on polyphenylalanine synthesis and the determination of the Mg^{2+} concentration optimum for the oviduct factor. Activity was assayed as described in Methods. (A) eIF-2A assay; a blank value of 1.4 pmol (minus eIF-5 and eIF-4 C and D) was subtracted. (B) Mg^{2+} concentration optimum; a saturating amount of aIF-2A (1.2 μg) was added.

of the elongation factors and initiation factors eIF-5 and eIF-C and D, the Mg^{2+} optimum was increased to 9–10 mM. The oviduct factor was capable, therefore, of lowering the Mg^{2+} concentration for optimum protein synthesis. Similar results have been observed with rabbit reticulocyte and liver initiation factors, eIF-2A (Shafritz & Anderson, 1970; Picciano et al., 1973).

Hen oviduct aIF-2A was capable of promoting the binding of the initiator tRNA to the 40S ribosomal subunit in a manner similar to the reticulocyte factor eIF-2A (Shafritz et al., 1972). Binding initially increased linearly with the addition of aIF-2A. Under these conditions aIF-2A bound 0.25 pmol of [^{3}H]-fMet-tRNA$_f$ per μg of protein per 3 min which was a fivefold increase over background activity. The results of assays which defined the dependencies and properties of the oviduct factor are given in Table I. The binding activity was dependent on the presence of 40S ribosomal subunits and the factor. Fifty percent of the initiator tRNA binding was dependent on the AUG codon. Like the activity of the reticulocyte factor, significant stimulation was observed with the addition of the initiator factors eIF-5 and eIF-4C. As previously reported for this binding activity in other systems there was no specificity

TABLE I: Requirements for aIF-2A-Directed Binding of Initiator tRNA to Reticulocyte 40S Ribosomal Subunits.[a]

Additions or deletions	[^{3}H]fMet-tRNA (pmol)
Complete (aIF-2A, eIF-5, eIF-4C and D, AUG, GTP)	0.25
−aIF-2A	0.05
−40S subunits	0.02
−AUG	0.10
−eIF-5, eIF-4C, and D	0.06
−eIF-5	0.12
−eIF-4C and D	0.10
−GTP	0.22

[a] Assay conditions were described in Methods. Each 50 μL of reaction mixture contained 7.5 pmol of [^{3}H]fMet-tRNA$_f$ (2930 cpm/pmol) and the other components except where indicated. All factors were added in saturating amounts: aIF-2A (2.0 μg), eIF-5 (0.6 μg), and eIF-4C and D (0.6 μg).

demonstrated for the nonformylated species of the initiator tRNA.

Since the oviduct factor was capable of directing the codon specific binding of the initiator tRNA to the 40S ribosomal subunit, the nature and specificity of the ribosomal binding site were next determined. The initiator tRNA is bound so that when the 80S ribosome is subsequently formed the tRNA occupies the ribosomal peptidyl site and the initial peptide bond is formed without translocation. The synthesis and release of the dipeptide, phenylalanyl-puromycin, at a low Mg^{2+} concentration are assays of this initial binding event. Oviduct aIF-2A stimulated the synthesis of [^{14}C]phenylalanyl-puromycin in a similar manner to reticulocyte eIF-2A in this assay (Figure 2). Both oviduct and reticulocyte factors stimulated the system four- to fivefold over background activity when added in saturating amounts.

Similar results were obtained measuring the AUG-dependent formation of the dipeptide, methionyl-puromycin, with the oviduct and reticulocyte factors. The total activity, however, was reduced about tenfold in this assay (data not shown).

Physical Properties of aIF-2A. The molecular weight was estimated by gel filtration chromatography. The elution volume of oviduct aIF-2A was determined by enzyme assay during gel filtration chromatography on an analytical, calibrated Sephadex G-200 column. Comparing this volume with those of standard protein markers, a molecular weight of 110 000 was estimated for aIF-2A. This was in agreement with the value of 95 000 obtained for the reticulocyte by a similar technique (Merrick & Anderson, 1975). Analyzing this data by the method of Laurent & Killander (1964), the Stokes radius of oviduct aIF-2A was determined to be 42.5 Å. This was similar to the value of 42 Å determined for the reticulocyte factor (Merrick & Anderson, 1975).

Sucrose Density Gradient Centrifugation. The sedimentation rate of aIF-2A activity was compared with those of standard protein markers in linear sucrose density gradients. The sedimentation coefficient of aIF-2A was determined to be 3.5 S compared with the reported value of 3.4 S for the reticulocyte factor (Merrick & Anderson, 1975). The molecular weight was calculated with the $s_{20,w}$ and Stokes radius to be 62 500 using the formula $M = 6\pi\eta Nas/(1 - \bar{v}\rho)$ where M is the molecular weight, η, viscosity, N, Avogadro's number, s, sedimentation coefficient, a, Stokes radius, $\bar{v}$, partial specific volume (0.732 cm^3/g), and ρ, solvent density (Siegel & Monty,

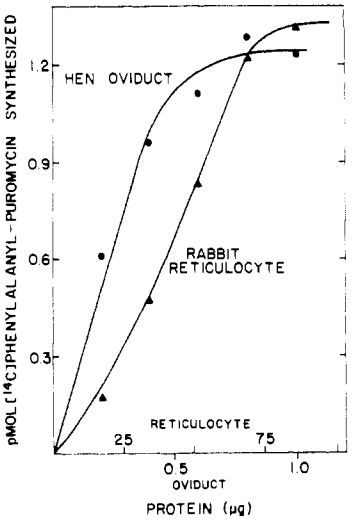

FIGURE 2: Stimulation of [^{14}C]phenylalanyl-puromycin synthesis by hen oviduct aIF-2A and rabbit reticulocyte eIF-2A. Assay conditions were described in Methods. Saturating amounts of reticulocyte EF-1, EF-2, and eIF-5 and eIF-4 C and D were added.

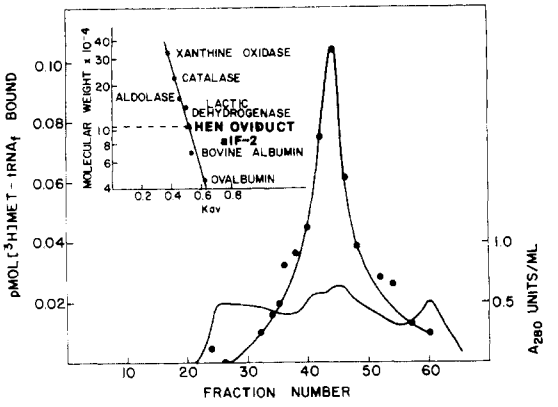

FIGURE 3: Ultrogel AcA-34 chromatography of hen oviduct aIF-2. Chromatographic methods and assay procedures were described in Methods. Twenty-microliter aliquots were assayed for binding activity. A blank value of 0.058 pmol was obtained in the absence of added protein and was subtracted. Elution volumes of molecular weight standards (10 mg of protein in 2 mL) were determined by absorbance at A_{280} as described in Methods.

1966). Similarly, with this molecular weight and other data the frictional ratio (f/f_0) was calculated to be 1.6 using the formula: $f/f_0 = a[4\pi N/3M(\bar{v} + \delta/\rho)]^{1/3}$ where δ is a solvation factor assumed to be 0.2 g/g of protein. The frictional ratio of the oviduct aIF-2A was in agreement with the value of 1.5 calculated for reticulocyte eIF-2A (Merrick & Anderson, 1975).

Isolation of aIF-2. The proteins of the oviduct nuclear microsomal salt wash absorbed to the DEAE-cellulose column were eluted with a linear salt gradient (Hejtmancik & Comstock, 1976). Fractions were assayed for an activity which was capable of binding Met-tRNA$_f$ in the presence of GTP to a nitrocellulose filter. Recently, a factor with this activity has been found in the absorbed fraction. It eluted between 125–160 mM KCl, therefore displaying similar ion-exchange chromatographic behavior to reticulocyte eIF-2 which eluted at 125–170 mM KCl (Safer et al., 1975a).

The oviduct binding factor, designated aIF-2, has been further purified by molecular sieve chromatography on Ultrogel AcA-34 (Figure 3). The Met-tRNA$_f$ binding activity eluted from this column as a single peak and the apparent

TABLE II: Characteristics of Aminoacyl-tRNA Binding by Oviduct aIF-2 and Reticulocyte eIF-2.[a]

Additions or deletions	[^{3}H]Met-tRNA$_f$ (pmol)	[^{14}C]Phe-tRNA (pmol)
aIF-2 + GTP	1.34	0.90
aIF-2 − GTP	1.20	0.70
eIF-2 (DEAE) + GTP	1.75	
eIF-2 (DEAE) − GTP	1.63	
eIF-2 (G-200) + GTP	1.77	0.16
eIF-2 (G-200) − GTP	0.34	0.10

[a] Assay conditions were described in Methods. Each 50 μL of reaction mixture contained 10.0 pmol of [^{3}H]Met-tRNA$_f$ (4930 cpm/pmol) or 10.0 pmol of [^{14}C]Phe-tRNA (1040 cpm/pmol). All factors were added in saturating amounts: aIF-2 (75 μg), eIF-2 (DEAE) (12 μg), and eIF-2 (G-200) (3.5 μg). Reticulocyte eIF-1 preparations designated DEAE and G-200 refer to steps 3 and 5, respectively, of the purification procedure (Safer et al., 1975a).

molecular weight of the oviduct factor was 102 000 as determined by the technique. Molecular weights of 90 300 and 175 000 were obtained for the homogeneous reticulocyte factor by molecular sieve chromatography depending on the concentration of protein applied to the column (Safer et al., 1975a). The higher value was observed with larger amounts of protein and was thought to represent a dimer of the lower molecular weight monomer.

Phosphocellulose batch elution chromatography (400–800 mM KCl) was subsequently carried out in order to purify the oviduct factor further; the active fractions were concentrated by ultrafiltration.

Biological Properties of aIF-2. A more detailed kinetic analysis of Met-tRNA$_f$ binding was determined for the concentrated aIF-2 fraction. The amount of Met-tRNA$_f$ bound in the complex was a linear function of the amount of protein added and occurred at a rate of 0.025 pmol of [^{3}H]Met-tRNA$_f$ per μg of protein per 2 min.

Oviduct aIF2 purified by these procedures showed only a minimal dependence on GTP (Table II). It can be seen that rabbit reticulocyte eIF-2 purified by DEAE-cellulose chromatography alone and under these conditions also showed a similar lack of GTP dependence. Binding specificity for the initiator tRNA was shown for more highly purified reticulocyte eIF-2 as previously reported (Safer et al., 1975b) but only partially for oviduct aIF-2.

In addition to the binding reaction, oviduct aIF-2 was capable of stimulating the formation of the synthetic dipeptide, methionyl-puromycin, on twice salt-washed ribosomes programmed with AUG template. The synthesis of the dipeptide increased linearly with increasing amounts of aIF-2 up to 1.5 μg of protein at a rate of 1.0 pmol of [^{3}H]methionyl-puromycin synthesized per μg protein per 10 min (data not shown).

Discussion

Two distinct eukaryotic factors bind in vitro the initiator tRNA into a complex which is capable with the ribosomal subunit joining factors, eIF-5 and eIF-4C, of forming functional ribosomes. In the present studies these two factors, designated aIF-2A and aIF-2, have been isolated and partially purified in the hen oviduct. Previously, only two other laboratories have isolated both factors from a single tissue. Ochoa & co-workers (Zasloff & Ochoa, 1973) have prepared homogeneous eIF-2′ (prime) from the supernatant of *A. salina* embryos and more recently they have identified the second factor designated eIF-2 in the supernatant and ribosomal washes (Filipowicz et al., 1975). Merrick & co-workers (Merrick & Anderson, 1975; Safer et al., 1975a) have succeeded in purifying to homogeneity both of these factors from the rabbit reticulocyte. The characteristic reactions of initiation as well as the physicochemical properties of these purified factors have been extensively studied and models proposed for their actions in forming an initiation complex by different pathways (Safer et al., 1975b; Weissbach & Ochoa, 1976). Unfortunately, these detailed studies have not defined the in vivo roles of these two factors in protein synthesis.

Hen oviduct aIF-2A closely resembled factor eIF-2A found in the rabbit reticulocyte and liver during molecular sieve and ion-exchange chromatography as well as in its in vitro activities. The apparent molecular weight and Stokes radius estimated by molecular sieve chromatography were similar to the reticulocyte factor. Due to the highly asymmetrical shape of these molecules, however, this value was reduced when the sedimentation coefficient and Stokes radius were used for the calculation. The hen oviduct and rabbit reticulocyte factors were interchangeable in several initiation assay systems tested.

Initiation factor aIF-2A was active in binding both the prokaryotic and eukaryotic initiator tRNAs to the 40S ribosomal subunit. Binding of [^{3}H]fMet-tRNA$_f$ was increased by the addition of the template AUG but was not stimulated by GTP. When crude reticulocyte eIF-2A was used no difference in factor requirements was observed between [^{3}H]Met-tRNA$_f$ and [^{3}H]fMet-tRNA$_f$ binding (Elson et al., 1975), but, when purified eIF-2A was used, eIF-4C was required but not eIF-5 or GTP for [^{3}H]Met-tRNA$_f$ binding (Adams et al., 1975). Since oviduct aIF-2A, although highly purified, was not homogeneous the fMet-tRNA$_f$ binding assay requirements appeared to be more similar to those of the crude reticulocyte factor. The first step initiation complex was capable of forming an 80S initiation ribosome as determined by the synthesis and release of the dipeptide aminoacyl-puromycin.

The oviduct factor aIF-2 demonstrated the same chromatographic properties as the reticulocyte factor eIF-2. The molecular weight corresponded to that determined for the monomer form of the reticulocyte protein. Since the concentration of protein added to the molecular sieve column was approximately 1.0 mg/mL, dimer formation was retarded. Oviduct aIF-2 was capable of binding initiator tRNA into a complex that was retained on nitrocellulose filters. It was presumed that it was a binary complex since its formation occurred independently of GTP. This lack of GTP dependence was disturbing except that similar results were obtained with crude reticulocyte eIF-2 (Elson et al., 1975; Safer et al., 1975b). The dependence on GTP appeared to increase as the purity of the reticulocyte factor increased in our studies but even with homogeneous eIF-2 a significant amount (50%) of Met-tRNA$_f$ binding occurred without GTP (Safer et al., 1975b). These investigators reported numerous factors and conditions which influenced the observed ratio of GTP-dependent to GTP-independent binding for eIF-2. Evidence indicated that the stable binary complex was formed by a factor designated IF-MP* (star) (eIF-2) which they believed was an altered state of the native molecule which no longer interacted with GTP. The binding activity might have been associated with a protein not involved in protein synthesis, for such a protein has been reported which bound nonspecifically aminoacyl-tRNAs in the absence of GTP (Gupta et al., 1973). However, this protein inhibited protein synthesis. The oviduct Met-tRNA$_f$ binding factor showed no inhibitory activity. aIF-2 was capable of forming a functioning initiation complex determined by the factor-dependent synthesis of the dipeptide, methionyl-puromycin.

Both avian oviduct factors aIF-2A and aIF-2, therefore, appeared similar in their physicochemical properties to the corresponding mammalian reticulocyte factors and functionally interchangeable with these factors as well. The similarities may be due to the techniques by which they were isolated since crude avian factors were screened for these specific activities with reticulocyte components.

Studies are in progress to purify further these factors which is necessary before a more precise definition of their roles in protein synthesis can be determined in this tissue. With additional information on the chemical and biological properties of aIF-2 in the oviduct it will be possible to determine whether the presence or absence of hormones controls its activity by phosphorylation of the protein in a mechanism similar to the regulation by hemin of globin synthesis (Clemens et al., 1975). The possibility that such a mechanism may exist is supported by the observations of Liang & Liao (1975) on binding Met-$tRNA_f$ to rat prostate ribosomes mediated by a prostate cytosol fraction. Binding activity decreased after castration but returned rapidly following the intravenous injection of dihydrotestosterone. Although the action of eIF-2A-like factors has not been defined and the suggestion made that it is an evolutionary remnant (Weissbach & Ochoa, 1976), our laboratory has recently observed that this factor alone is able to stimulate the initiation of endogenous messenger RNA translation on membrane-bound oviduct polysomes. The factor cannot, however, stimulate translation of exogenous mRNA on these polysomes or on reticulocyte free polysomes.

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Synthesis and Characterization of 2-Nitro-5-azidobenzoylglycyloxytocin, an Oxytocin Photoaffinity Label†

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ABSTRACT: The oxytocin analogue, 2-nitro-5-azidobenzoylglycyloxytocin (NAB-Gly-oxytocin), has been synthesized and purified. The analogue is a full agonist for the stimulation of osmotic water flow in the toad urinary bladder (one-half maximal activity at 3.2×10^{-6} M). It also enhances [^{14}C]urea permeability in this tissue. Repetitive photolysis in the presence of NAB-Gly-oxytocin (8×10^{-6} M) results in a progressive permanent inhibition of oxytocin stimulated urea permeability but does not alter hormone induced ^3H_2O movement. The inhibition is dependent on the photogeneration of the aryl nitrene intermediate and is relieved by protecting the hormone receptor with excess oxytocin (10^{-6} M) during the photolysis. These results suggest that the photodependent permanent inhibition of the response to oxytocin in the toad bladder is due to covalent incorporation of the photoaffinity label, NAB-Gly-oxytocin, into the hormone receptor.

The initial event in the action of peptide hormones is the binding of these ligands to specific receptors in the plasma membrane of target tissue cells (Cuatrecasas, 1974; Kahn, 1975). Recently there has been increasing interest in the identification, characterization, and isolation of these receptors. Several peptide hormone receptors have been identified by observing the specific, reversible binding of radioactively labeled hormones in isolated cells and membrane preparations from target tissues (Kahn, 1975; Catt & Dufau, 1977). However, even during mild purification procedures an alteration and/or loss of the specific binding properties for the ligand is observed as the receptor is solubilized away from the membrane lipid bilayer. Additionally, receptor solubilization often uncouples the receptor from any functional marker, e.g., adenylate cyclase, that could be followed through successive purification steps. It is necessary, therefore, to devise means of labeling hormone receptors covalently, in situ, and follow the incorporated label as a means of monitoring the isolation procedures.

The application of photoaffinity labeling to the problem of biological receptor identification has been discussed (Knowles, 1972; Cooperman, 1976). Photoaffinity labeling has a distinct advantage over chemical affinity labeling since the interaction of the ligand analogue with the receptor of interest can be characterized prior to covalent incorporation (dark experiments). The highly reactive intermediates, usually carbenes or nitrenes, generated in the binding site during photolysis increase the potential for specific labeling of the receptor, even in complex biological systems. Photoaffinity labeling has found wide application in the investigation of enzymes (Singh et al., 1962; Shafer et al., 1966), antibody binding sites (Fleet et al., 1972; Yoshioka et al., 1973; Choy et al., 1975; Converse & Richards, 1969), and nucleotide receptors (Guthrow et al., 1973; Haley, 1975; Pomerantz et al., 1975; Brunswick & Cooperman, 1971; Schafer et al., 1976).

In the present study we report the synthesis and purification of the photoactivated hormone analogue 2-nitro-5-azidobenzoylglycyloxytocin (NAB-Gly-oxytocin). The hormone oxytocin was chosen for these studies because its amino acid sequence (Figure 1) allows controlled derivatization and large quantities of the synthetic peptide were available. Additionally, the design of the photolabile hormone analogue was greatly facilitated by the availability of extensive structure-activity data (Rasmussen et al., 1963; Sawyer & Manning, 1973; Rudinger et al., 1972; Walter et al., 1971, 1967; Walter, 1971). These structure-activity studies made it clear that coupling of the photoactive reagent to the N-terminal amine of oxytocin would bring the aryl azide in close contact with the receptor, but not completely interfere with the binding specificity of the receptor for the hormone. We found that NAB-Gly-oxytocin retained a high affinity for the antidiuretic hormone receptor in the isolated toad urinary bladder. The derivatized hormone was a potent agonist for the stimulation of osmotic water flow (one-half maximal activity at 3.2×10^{-6} M) as well as stimulating [^{14}C]urea diffusion in this tissue. Photolysis of the toad bladder in the presence of NAB-Gly-oxytocin led to a permanent inhibition of the oxytocin enhanced change in [^{14}C]-urea permeability. Evidence that this permanent inhibition is due to covalent labeling of the antidiuretic hormone receptor by the photoactivated oxytocin analogue is provided by the fact that this inhibition is dependent on the presence of the azido

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