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Germination of *Dictyostelium discoideum* Spores. A ^{31}P NMR Analysis†

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ABSTRACT: Perchloric acid extracts of *Dictyostelium* spores have been investigated by ^{31}P nuclear magnetic resonance (NMR) spectroscopy. This analysis has allowed the assignment of all the ^{31}P resonances observed in vivo to specific compounds. Dormant spores have been found to contain as prominent phosphorylated metabolites two phosphomonoesters, phosphoethanolamine and inositol hexakis(phosphate), two phosphodiesters, glycerophosphocholine and glycerophosphoethanolamine, as well as nucleoside triphosphates and polyphosphates. The very large amounts of glycerophosphocholine, glycerophosphoethanolamine, and phosphoethanolamine in spores were the most remarkable differences from *Dictyostelium* amoebae. In vivo ^{31}P NMR has shown that the peak of nucleoside triphosphates in dormant spores was maintained metabolically since it disappeared completely upon anaerobiosis. The pH-sensitive ^{31}P NMR signal of phosphoethanolamine was used to determine internal pH, and a value of pH 6.5 was found in aerobic *Dictyostelium* dormant spores. Spore germination, induced by activation with heat shock treatment, was monitored noninvasively by ^{31}P NMR. No change in phosphorylated components was observed to have occurred during the activation step. The major modifications in phosphorylated metabolites observed upon germination of the activated spores were the progressive disappearance of the two phosphodiesters glycerophosphocholine and glycerophosphoethanolamine.

The differentiation program of the cellular slime mold *Dictyostelium discoideum* is engaged upon starvation and leads to the formation of a fruiting body composed of a mass of spores supported by a thin cellular stalk (Bonner, 1967; Loomis, 1975; Raper, 1984). Experimental parameters governing the germination process have revealed a complex and highly controlled mechanism (Cotter, 1981; Cotter & Raper, 1966, 1968a,b).

In this work, we have examined the phosphorylated metabolites of *Dictyostelium* spores in the dormant stage and during the whole germination process using the noninvasive ^{31}P NMR spectroscopy technique. We report that *Dictyostelium* spores contain very large amounts of two phosphodiesters: glycerophosphocholine (GPC)[1] and glycerophosphoethanolamine (GPE). These two compounds progressively disappeared during the germination of activated spores.

Experimental Procedures

Chemicals. Methylenediphosphonic acid, GPE, GPC, phosphoethanolamine, and phosphocholine were purchased from Sigma. Poly(propylene glycol) 2000 was from Baker Chemical.

Culture Conditions. Dictyostelium discoideum, strain NC4H (ATCC 34071), was grown at 22 ± 1 °C in association

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[1] Abbreviations: GPC, glycerophosphocholine; GPE, glycerophosphoethanolamine; IP3 *myo*-inositol 1,4,5-tris(phosphate); IP4, *myo*-inositol 1,3,4,5-tetrakis(phosphate); IP6, *myo*-inositol hexakis(phosphate) (phytic acid); Mes, 2-(*N*-morpholino)ethanesulfonic acid; PE, phosphatidylethanolamine; PC, phosphatidylcholine; P_i, inorganic phosphate.

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with the bacteria *Escherichia coli* B/r (Cotter & Raper, 1966) or with *Klebsiella aerogenes* (Sussman, 1966). Mature spores obtained 2–3 days after culmination were harvested by shaving the spore heads with the edge of glass slides, suspended in 10 mM Mes-K buffer, pH 6.5, and centrifuged at 1200g for 4 min at 22–23 °C. Spores were washed twice with 10 mM Mes-K buffer, pH 6.5.

NMR Spectroscopy of Spores. General ^{31}P NMR conditions were as described previously for *Dictyostelium* amoebae (Satre et al., 1986; Klein et al., 1988). Poly(propylene glycol) (M_r 2000) was added as antifoam agent. At the concentration used in this work, 10 μL of poly(propylene glycol) per 20 mL of cell suspension, no adverse biological effect was noted on *Dictyostelium* spores (or amoebae as well). Proton-decoupled spectra were recorded on a Bruker WM2000 WB spectrometer operating at 81 MHz for ^{31}P. Acquisition conditions used 60° (10-μs) radio-frequency pulses at 0.6-s intervals, and spectral width was 6024 Hz. Two levels of decoupling were used: 2.5 W for 0.34 s (acquisition time) and 0.5 W for 0.26 s. Other specific conditions are detailed in the legends to the figures. Free induction decays were obtained into 4K data points and processed with 5-Hz exponential line broadening.

Preparation of $HClO_4$ Extracts from Dictyostelium Spores. $HClO_4$ extracts were performed on 20 mL of spores (from 4 × 10^8 to 8 × 10^8 spores/mL) supplemented with 10 μL of poly(propylene glycol) and oxygenated for 15 min at 22 °C in 10 mM Mes-K buffer, pH 6.5, before addition of ice-cold $HClO_4$ to a final concentration of 1.3 M. The mixture, quickly frozen in the form of beads in liquid N_2, was transferred to a precooled mortar and crushed with a pestle to give a smooth powder which started to melt at about −15 °C. The extent of spore breakage was controlled by microscopical examination, and if required, freezing and crushing steps were repeated. French press or glass bead treatments were found inefficient for the breakage of spores in the presence of $HClO_4$, in contrast to their effect on spores suspended in buffer (Tisa & Cotter, 1980). Further steps were as described previously (Martin et al., 1987; Klein et al., 1988).

NMR Spectroscopy of $HClO_4$ Extracts from Dictyostelium Spores. Proton-coupled or -decoupled spectra were recorded on a Bruker AM400 spectrometer operating at 162 MHz for ^{31}P. Acquisition conditions used 60° (10-μs) radio-frequency pulses at 4- or 21-s intervals, and spectral width was 8475 Hz. For decoupled spectra, two levels of decoupling were used: 2.5 W for 0.48 or 1 s (acquisition time) and 0.5 W for 3.52 or 20 s. A repetition time of 21s gave fully relaxed signals. Other specific conditions are detailed in the legends to the figures. Free induction decays were obtained into 8K data points, zero-filled to 32K, and processed with 0.5-Hz exponential line broadening. For quantitative determinations, after recording the original ^{31}P NMR spectra, internal calibrations were performed by addition of known amounts of P_i.

Phosphoethanolamine pH Calibration Curve. The pH titration data of phosphoethanolamine were fitted to the equation:

$$pH = pK + \log (d - A)/(B - d)$$

where pK is the dissociation constant, d is the measured chemical shift at a given pH, and A and B are the limiting chemical shifts at acidic or basic pHs. The following values were obtained: $pK = 5.67 \pm 0.03$; $A = 0.44 \pm 0.06$; $B = 3.97 \pm 0.04$ (mean ± SEM; three experiments).

RESULTS

^{31}P NMR Spectrum of a Perchloric Acid Extract of Dormant Spores. A ^{31}P NMR spectrum of a $HClO_4$ extract prepared from aerobic *Dictyostelium* dormant spores is shown in Figure 1. Identifications in these extracts were made on the basis of several criteria such as chemical shift values and their pH dependence, and also by comparing resonance fine structure in proton-coupled and decoupled NMR spectra as well as by direct spiking with authentic compounds in the extracts. The phosphodiesters GPE and GPC were the two major resonances at 0.6–0.7 and 0–0.1 ppm, respectively. Their characteristic fine structure in a proton-coupled spectrum is shown in Figure 1E. Quantitatively, GPE and GPC represented about 25–30% of the free phosphorylated compounds in *Dictyostelium* spores, and the ratio GPC/GPE was close to 1.5.

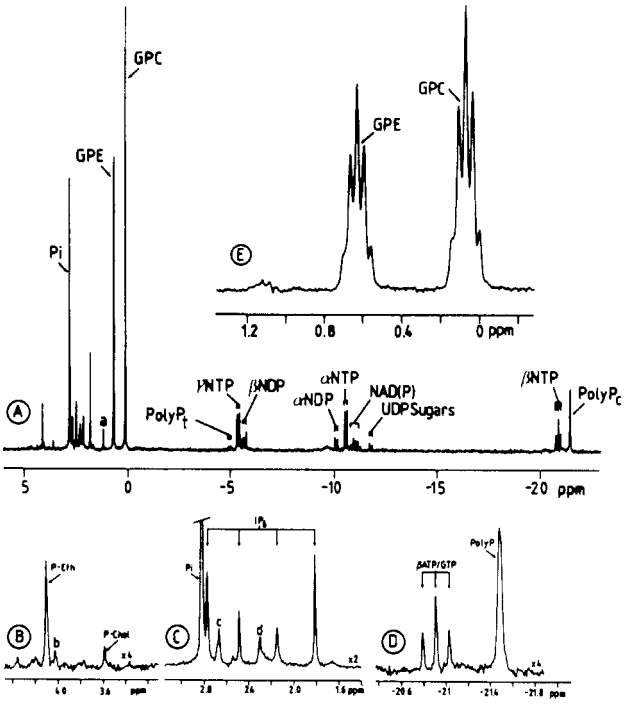

FIGURE 1: Typical ^{31}P NMR spectrum of a $HClO_4$ extract from *Dictyostelium* aerobic spores. The $HClO_4$ extract was prepared from a total of 1.6 × 10^{10} spores as described under Experimental Procedures and adjusted to pH 8.3 before NMR measurements. This proton-decoupled spectrum (A) was the sum of 3600 free induction decays with 4-s interpulse delays. Specific portions of the spectrum are shown on expanded scales in (B), (C), and (D). Enlargement E is a portion of another spectrum acquired without broad-band decoupling and showing the fine structure of GPE and GPC. Peak assignments were as follows: P-Chol, phosphocholine; P-Eth, phosphoethanolamine; P_i, inorganic phosphate; IP6, inositol hexakis(phosphate); GPC, glycerophosphocholine; GPE, glycerophosphoethanolamine; α, β, or γ NTP, unresolved α-P, β-P, or γ-P resonances of purine nucleoside triphosphates; β NDP, unresolved β-P resonances of purine nucleoside diphosphates; UDP sugars, uridine diphosphoglucose, -*N*-acetylglucosamine, or -galactose; NAD(P), α-P of NAD(P); PolyPt and PolyPc, terminal and central P atoms of polyphosphate chains. Minor resonances labeled a–d were tentatively assigned to (a) glycerophosphoinositol, (b) nucleoside monophosphates, and (c and d) lower inositol phosphates (these latter lines were split into doublets in a proton-coupled spectrum).

Other major components identified previously in *Dictyostelium* amoebae (Martin et al., 1987) such as P_i, IP6 (Figure 1C), and the various resonance lines of P atoms of nucleoside di- and triphosphates were clearly apparent. Interestingly, only purine nucleoside triphosphates (ATP/GTP) were represented in significant amounts in spores, and the amount of pyrimidine nucleoside triphosphates (CTP/UTP) was at least 15–20 times lower. Similar results were reported previously by using HPLC of extracts from dormant spores (Hamer & Cotter, 1983). This was not the case for vegetative

Table I: Intracellular Concentrations (mM) of Phosphorylated Metabolites in Aerobic Dormant *Dictyostelium* Spores[a]

phosphoethanolamine	4.8 ± 1.2	GPE	18.1 ± 1.1
phosphocholine	1.0 ± 0.4	GPC	26.7 ± 1.1
P_i	13.6 ± 0.6	NDP	1.6 ± 0.1
IP6	2.7 ± 0.2	NTP	4.5 ± 0.4
GPI	1.1 ± 0.2	polyphosphates[b]	24 ± 9

[a] Results are means ± SEM (n = 3). The listed compounds represented about 75% of the total integration of the spectrum. [b] This value corresponded to an equivalent P_i concentration. The corresponding average length of these polyphosphate chains was 24 ± 6 P atoms.

amoebae where the purine/pyrimidine ratio was 2.7 (Satre et al., 1986) or for newborn amoebae arising from germinated spores (not shown).

The resonances from the central P atoms of polyphosphates were present at −21.5 ppm. The average chain length of these NMR-visible polyphosphates in Figure 1 was calculated to be about 25 P residues on the basis of the intensity of the resonance lines near −5 ppm arising from the terminal phosphate atoms of polyphosphates, relative to the intensity of the resonance at −21.5 ppm arising from central phosphate atoms. The phosphomonoesters region showed one main component identified as phosphoethanolamine (at +4.1 ppm) and a 5-fold smaller amount of phosphocholine (at +3.6 ppm).

Metabolite Concentrations in Dormant Spores. The ^{31}P NMR spectra of the $HClO_4$ extracts from dormant spores used for quantitation were acquired under fully relaxed conditions and without decoupling to avoid interference with nuclear Overhauser enhancement (NOE). Under these conditions, the integrated intensities of the NMR peaks were proportional to their concentrations. The mean spore volume of *Dictyostelium* NC4H was determined with the Coulter counter and was found to be 39 ± 1 μm^3 (n = 7). This value was in good agreement with the spore dimensions of *Dictyostelium* NC4 given by Raper (1984) although larger values were reported by Loomis (1975). From the measured spore volume, the intracellular concentrations of several phosphorylated metabolites are given in Table I.

In Vivo ^{31}P NMR Spectra of Aerobic Dormant Spores. Figure 2 shows a ^{31}P NMR spectrum of an oxygenated suspension of *Dictyostelium* dormant spores. Major resonance peaks corresponded to the expected positions of the central P atoms of long-chain polyphosphates at −22.8 ppm and to the various P atoms of nucleoside di- and triphosphates at −19.2, −10.4, and −5.5 ppm. The NTP/(NTP + NDP) ratio was close to 75%, a value similar to the one measured earlier in vegetative amoebae (Satre et al., 1986). The β-P resonance characteristic of NTP at −19.2 ppm required strictly aerobic conditions to be present. It disappeared completely upon anaerobiosis (Figure 2, inset) and was restored upon subsequent reoxygenation. The broad cluster between +4 and −1 ppm corresponded to the resonance positions expected for phosphodiesters, P_i, IP6, and other phosphomonoesters. The compounds giving rise to the two sharp resonances in this region at +0.09 and +0.62 ppm were likely to arise from the presence of large amounts of the two phosphodiesters, GPC and GPE, identified in the $HClO_4$ extracts (see Figure 1). The sharp resonance lines were indicative of rapidly tumbling molecules. The most downfield resonance at +3.6 ppm corresponded mainly to phosphoethanolamine.

Some variability (2- to 3-fold) was noted in the polyphosphate levels in *Dictyostelium* spores, as reported previously (Gezelius, 1974). Identical profiles were observed with spores arising from amoebae fed either with *E. coli* B/r or *K. aerogenes*. The spectrum of dormant spores remained stable upon prolonged incubation under both aerobic and anaerobic

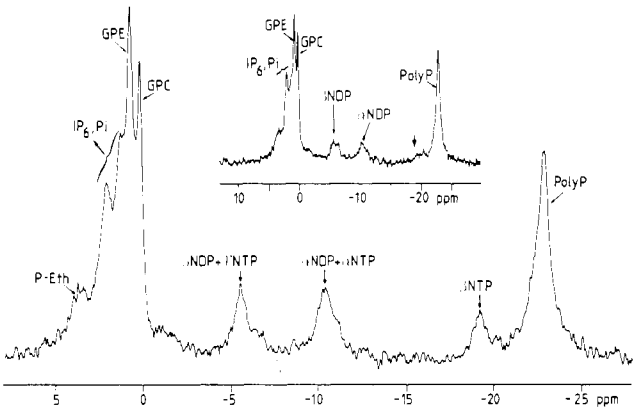

FIGURE 2: ^{31}P NMR spectrum of *Dictyostelium* dormant spores. Data were accumulated from a total of 1.2×10^{10} spores oxygenated in 20 mL of 10 mM Mes-K buffer, pH 6.5. This proton-decoupled spectrum was the sum of 3000 free induction decays with 0.6-s interpulse delays. The inset shows a spectrum of dormant spores bubbled with nitrogen. The arrow points to the position expected for the β-P NTP resonance.

conditions (up to 14 h) although some release of P_i and also polyphosphates occurred into the external medium.

The proportion of free NTP, in *Dictyostelium* spores, calculated from the difference between the chemical shifts of the β and α resonances of NTP measured in vivo (Gupta et al., 1984), was 15 ± 3% (n = 8). Assuming a dissociation constant of 0.05 mM for the NTP–Mg^{2+} complex, a value of 0.3 ± 0.1 mM was found for the concentration of free Mg^{2+} in dormant spores. This concentration was similar to the one determined previously in vegetative amoebae (Satre & Martin, 1985).

Measurement of Spore Internal pH. A single metabolite, phosphoethanolamine, represented more than 80% of all the dormant spore phosphomonoesters resonating lowfield of P_i (Figure 1). This intracellular compound was thus used as a probe of spore internal pH as the position of the resonance of intracellular P_i was expected to occur in a region of the in vivo ^{31}P NMR spectrum of *Dictyostelium* spores blurred by the resonances of GPC, GPE, and IP6. Considering the position of an in vivo phosphoethanolamine resonance which appeared as the leftmost shoulder at +3.6 ± 0.1 ppm (n = 5), the internal pH in aerobic dormant spores was found to be pH 6.5 ± 0.1. A negligible acidification occurred under anaerobic conditions (Figure 2, inset).

Spore Germination. The evolution of the phosphorylated metabolites detected in the ^{31}P NMR profile of spores in an oxygenated suspension was followed after activation by the heat shock treatment (Cotter & Raper, 1966, 1968a). As shown in Figure 3, the first spectrum obtained after heat activation was very similar to that of dormant spores (Figure 2).

In the subsequent spectra, the most significant changes were a progressive decrease of GPC and GPE peaks. The total signal intensity decreased progressively, suggesting that GPE and GPC were converted into NMR-invisible components, most likely phospholipids. During germination, modifications in the successive ^{31}P NMR spectra also occurred at the level of polyphosphates as the resonance at −22.8 ppm increased slightly in intensity and its width at half-height decreased continuously from 120 Hz immediately after heat activation down to 60–70 Hz after 5 h of incubation. These variations were most likely the result of the mobilization of some of the NMR-invisible polyphosphates and of a progressive shortening of the polyphosphate chains. The NTP/(NTP + NDP) ratio and free Mg^{2+} both remained stable during germination and at levels similar to that of dormant spores.

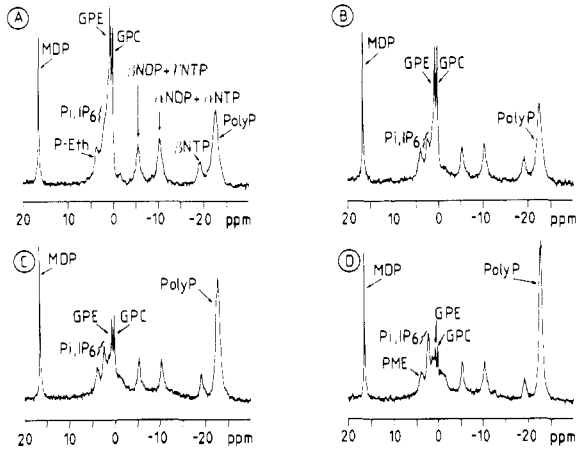

FIGURE 3: Germination of heat-activated *Dictyostelium* spores. Washed spores (1.2×10^{10}) suspended in 5 mL of 10 mM Mes-K buffer, pH 6.5, were incubated for 30 min at 45 °C and centrifuged for 4 min at 1200*g*. The activated spore pellet was immediately suspended in 20 mL of the above buffer at 22 °C, and consecutive ^{31}P NMR spectra were recorded. Each proton-decoupled spectrum was the sum of 3000 free induction decays with 1.2-interpulse delays and corresponded to the following periods after activation: (a) 0–1 h; (b) 1–2 h; (c) 2–3 h; (d) 3–4 h. MDP, resonance of methylenediphosphonate in a capillary used as an external reference.

The entire process was also observed to take place in the presence of a protein synthesis inhibitor, cycloheximide (0.5 mg/mL), although in this case the spores became activated and swollen but no emergence took place (Cotter & Raper, 1966, 1970).

Germination of *Dictyostelium* was shown earlier to be strictly aerobic (Cotter & Raper, 1968a; Kobilinski & Beattie, 1977), and oxygenation was essential to observe all the NMR-detected metabolic changes that occurred concomitantly to the swelling of the spores until emergence of amoebae. If heat-activated spores were bubbled with nitrogen, GPE and GPC remained at their high level and no ^{31}P NMR detectable metabolic evolution occurred.

Discussion

A major finding of these studies on *Dictyostelium* spores was the observation of large amounts of two phosphodiesters, GPE and GPC, which accounted for more than 25% of the total phosphate containing metabolites measured by ^{31}P NMR. This was in contrast to amoebae where previous NMR studies have shown that they contain very low levels of GPE and GPC (Jentoft & Town, 1985; Kay et al., 1986; Martin et al., 1987). GPE and GPC have been reported to be present in a broad range of cells and sometimes in quite high concentrations, but their metabolic role remains unclear (Burt, 1985). The major phospholipid components of *Dictyostelium* amoebae were shown to be PC (30%) and PE (45%) (Ellingson, 1974; Weeks & Herring, 1980). The idea that GPE and GPC found in *Dictyostelium* spores were the degradation products of PE and PC, respectively, was supported by evidence for very high phospholipase A and lysophospholipase activities in *Dictyostelium* amoebae (Ferber et al., 1970; Irvine et al., 1980; Ellingson & Dischinger, 1984). Fatty acids produced by sequential hydrolysis of phospholipids could ensure a source of energy during development in complement to that derived from amino acid and sugar catabolism (Cotter & Raper, 1970; Kelleher et al., 1979). The phosphodiesters GPE and GPC may also serve at some developmental stage as endogenous lysophospholipase inhibitors to preserve membrane phospholipids in cell structures during the formation of spores (Burt, 1985). The decrease of both GPE and GPC observed during germination could correspond to an expected increased activity of membrane phospholipid synthesis during this period.

Dormant spores, similarly to amoebae (Martin et al., 1987; Europe-Finner et al., 1988), contained large amounts of IP6 whose function is still unclear. The process of synthesizing IP6 is metabolically expensive, involving several ATP-requiring steps. This could be a way for the cell to escape Ca^{2+}-releasing stimulation arising from the presence of metabolically active isomers of IP3 and IP4. Sequential phosphorylations of these lower inositol polyphosphates to produce IP6 could provide an alternative to the phosphatasic reactions hydrolyzing IP3 or IP4 down to free inositol (Berridge, 1987). IP6 was not used up appreciably during spore germination, excluding its direct role as a store of phosphorus or high-energy phosphoryl groups. As IP6 forms high-affinity complexes with Mg^{2+} or Ca^{2+}, a possible function as buffering element in the regulation of intracellular divalent cations concentrations should also be considered.

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Registry No. P_i, 14265-44-2; IP6, 83-86-3; GPI, 16824-65-0; GPE, 1190-00-7.

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Natural mRNA Is Required for Directing Met-tRNA$_f$ Binding to 40S Ribosomal Subunits in Animal Cells: Involvement of Co-eIF-2A in Natural mRNA-Directed Initiation Complex Formation†

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ABSTRACT: Two protein factors, eIF-2 as well as a high molecular weight protein complex from reticulocyte ribosomal high-salt wash which we term Co-eIF-2, promote Met-tRNA$_f$ binding to 40S ribosomes. This binding is dependent on the presence of an AUG codon or natural mRNAs [Roy et al. (1984) *Biochem. Biophys. Res. Commun. 122*, 1418–1425]. Co-eIF-2 contains two component activities, Co-eIF-2A and Co-eIF-2C. Previously, we have purified an 80-kDa polypeptide containing Co-eIF-2A activity and showed that this polypeptide is a component of Co-eIF-2 and is responsible for Co-eIF-2A activity in Co-eIF-2 [Chakravarty et al. (1985) *J. Biol. Chem. 260*, 6945–6949]. We now report purification of a protein complex (subunits of M_r 180K, 110K, 65K, 63K, 53K, 50K, 43K, and 40K) containing Co-eIF-2C activity and devoid of Co-eIF-2A activity. In SDS–PAGE, the purified Co-eIF-2C preparation and an eIF-3 preparation (purified in Dr. A. Wahba's laboratory) separated into seven similar major polypeptides (M_r 110K, 65K, 63K, 53K, 50K, 43K, and 40K). The 50-kDa polypeptide in Co-eIF-2C was immunoreactive with a monoclonal antibody against eIF-4A (50 kDa). We have studied the roles of purified Co-eIF-2A and Co-eIF-2C activities in ternary and Met-tRNA$_f$·40S ribosome complex formation. The results are as follows: (1) At low and presumably physiological factor concentration (30 nM), eIF-2 did not form detectable levels of ternary complex. Moreover, such complex formation was totally dependent on the presence of Co-eIF-2A and/or Co-eIF-2C. (2) Only the ternary complex formed with Co-eIF-2A was stable in the presence of physiological concentrations of natural mRNAs. (3) Co-eIF-2C but not Co-eIF-2A contained guanine nucleotide exchange factor (GEF) activity. (4) In the presence of Mg^{2+} and natural mRNAs, the ternary complex formation by eIF-2 was completely dependent on the presence of both Co-eIF-2A and Co-eIF-2C. Co-eIF-2C is presumably required to promote GDP displacement from eIF-2·GDP while Co-eIF-2A is required to stabilize the ternary complex toward natural mRNAs. (5) The combined presence of eIF-2 together with Co-eIF-2C efficiently promoted AUG-dependent Met-tRNA$_f$·40S ribosome complex formation. However, Co-eIF-2A was required besides eIF-2 + Co-eIF-2C for Met-tRNA$_f$·40S ribosome complex formation dependent on a natural mRNA. Several mRNAs, including globin mRNA, polio RNA, brome mosaic viral RNA, and cowpea mosaic viral RNA, actively stimulated (3–6-fold) Met-tRNA$_f$ binding to 40S ribosomes under the assay conditions. A mechanism for the early steps in peptide chain initiation leading to Met-tRNA$_f$·40S·mRNA complex formation has been proposed.

The first step in peptide chain initiation in animal cells is the formation of a ternary complex between Met-tRNA$_f$, the eukaryotic peptide chain initiation factor 2 (eIF-2)[1] and GTP: Met-tRNA$_f$·eIF-2·GTP. The next step is the transfer of Met-tRNA$_f$ to 40S ribosomes in the presence of mRNA and formation of the Met-tRNA$_f$·40S·mRNA complex. Two outstanding questions in this study and addressed in this paper are related to (1) characteristics and requirements of the peptide chain initiation factors in Met-tRNA$_f$·40S·mRNA complex formation. Several laboratories have reported requirements of multiple protein factors for such complex formation. However, there are significant controversies regarding the characteristics of the protein factors and their roles in peptide chain initiation [for recent reviews, see Ochoa (1983), Moldave (1985), Proud (1986), Gupta (1987), and Gupta et al. (1987)]. (2) The requirement of mRNA for Met-tRNA$_f$ binding to 40S ribosomes is the second question. Earlier studies in several laboratories using crude peptide chain initiation factor preparations have indicated that Met-tRNA$_f$

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[1] Abbreviations: eIF-2, eukaryotic peptide chain initiation factor 2, which forms the ternary complex Met-tRNA$_f$·eIF-2·GTP; Co-eIF-2, a high molecular weight protein complex which contains Co-eIF-2A and Co-eIF-2C activities; Co-eIF-2A, stimulates ternary complex formation and also stabilizes the complex toward physiological mRNA; Co-eIF-2C, promotes GDP displacement from eIF-2·GDP in the presence of Mg^{2+} and also stimulates ternary complex formation; GEF, guanine nucleotide exchange factor which promotes GDP displacement from eIF-2·GDP in the presence of Mg^{2+}; SDS, sodium dodecyl sulfate; PAGE, polyacrylamide gel electrophoresis; BMV, brome mosaic viral RNA; CPMV, cowpea mosaic viral RNA.

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