Influence of the state of ribosome association on the phosphorylation of ribosomal proteins in isolated ribosome–protein kinase systems from rat cerebral cortex

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Ribosomal protein phosphorylation was investigated in isolated ribosomal subunits and polyribosomes from rat cerebral cortex in the presence of [γ - 32 P]ATP and purified catalytic subunit of cyclic AMP-dependent protein kinase from the same tissue. Ribosomal proteins that were most readily phosphorylated in isolated cerebral ribosomal subunits included proteins S2, S3a, S6 and S10 of the 40S subunit and proteins L6, L13, L14, L19 and L29 of the 60S subunit. These proteins were also phosphorylated in cellular preparations of rat cerebral cortex *in situ* or *in vitro* [Roberts & Ashby (1978) *J. Biol. Chem.* **253**, 288–296; Roberts & Morelos (1979) *Biochem. J.* **184**, 233–244]. However, several additional ribosomal proteins were phosphorylated when isolated 40S or 60S subunits were separately incubated in the reconstituted system. Analogous results were obtained with an equimolar mixture of cerebral 40S and 60S subunits under comparable conditions. In contrast, extensive exposure of purified cerebral polyribosomes to the catalytic subunit resulted in phosphorylation of only those ribosomal proteins of the 40S subunit that were most highly labelled after the administration of [32 P]P_i *in vivo*: proteins S2, S6 and S10. Ribosomal proteins of 60S subunits that were readily phosphorylated in isolated cerebral polyribosomes included proteins L6, L13 and L29. These results indicate that polyribosome formation markedly decreases the number of ribosomal protein sites available for phosphorylation by the catalytic subunit of cyclic AMP-dependent protein kinase. Moreover, the findings suggest that, of the ribosomal protein phosphorylations observed in rat cerebral cortex *in vivo*, proteins S2, S6, S10, L6, L13 and L29 can be phosphorylated in polyribosomes, whereas proteins S3a, S5, L14 and L19 may become phosphorylated only in free ribosomal subunits.

Numerous recent investigations have dealt with the regulation of ribosomal protein phosphorylation in eukaryotic cells and the relationship of this phenomenon to ribosomal function (for reviews see Wool, 1979; Leader, 1980; Traugh, 1981). However, a clear understanding of these phenomena has not emerged. The limited number of ribosomal protein phosphorylations that occur in rat cerebral cortex *in situ* have been well characterized (Roberts & Morelos, 1979). In common with the results of studies with other eukaryotic cells and tissues, elevated concentrations of cyclic AMP enhanced phosphorylation of protein S6, the major phosphoprotein of the cerebral 40S ribosomal subunit, but appeared to be without effect on the phosphorylation of other ribosomal proteins (Roberts &

Ashby, 1978; Roberts & Morelos, 1979). Cyclic GMP had no obvious effect on ribosomal protein phosphorylation *in vivo*. These findings, as well as investigations from other laboratories, suggested that phosphorylation of ribosomal proteins other than protein S6 might normally be directed by cyclic nucleotide-independent protein kinases (Traugh, 1981). However, purified preparations of cyclic nucleotide-regulated protein kinases, incubated with isolated 40S and 60S ribosomal subunits from a variety of eukaryotic cells, are capable of stimulating phosphorylation of all of the ribosomal proteins phosphorylated *in vivo* as well as numerous other ribosomal proteins (Eil & Wool, 1973a; Ventimiglia & Wool, 1974; Francis & Roberts, 1978, 1980; Issinger *et al.*, 1980). Since ribosomal

subunits are normally scarce in eukaryotic cells, these results suggest that conformational restraints and other processes that accompany ribosomal subunit association *in vivo* may be important determinants of the physiological spectrum of ribosomal protein phosphorylations. The present experiments were undertaken to determine whether the ribosomal protein phosphorylations observed in rat cerebral cortex *in situ* could be reproduced in isolated systems by the controlled action of cerebral cyclic AMP-dependent protein kinase on cerebral ribosomes in various states of association.

Experimental

Preparation of cerebral ribosomes and ribosomal subunits

Cerebral-cortical ribosomes were isolated from young rats (14–20 days old) of an inbred strain of Sprague–Dawley rats (Roberts & Morelos, 1979). Purified ribosomal subunits and polyribosomes larger than the trimer were prepared from these salt-washed ribosomes as described in detail previously (Roberts & Morelos, 1980). These preparations were free of detectable phosphoprotein phosphatase or protein kinase activities measured as outlined below. They were either used immediately in phosphorylation experiments or stored at -70°C for subsequent use.

Isolation of the catalytic subunit of cerebral cyclic AMP-dependent protein kinase

The catalytic subunit of cyclic AMP-dependent protein kinase II was isolated from rat cerebral cytosol essentially as described by Bechtel *et al.* (1977). The post-mitochondrial-supernatant fraction from pooled rat cerebral cortices was centrifuged at 226 600 *g* for 1–1.5 h at 4°C in a Beckman 50Ti rotor. The resulting postmicrosomal supernatant fraction was adjusted to 70% saturation with $(\text{NH}_4)_2\text{SO}_4$. Precipitated protein was recovered by centrifugation at 120 000 *g* for 15 min at 4°C , taken up in buffer containing 20 mM-Tris/HCl (pH 7.6 at 4°C)/15 mM-2-mercaptoethanol, and batch-adsorbed on preswollen DEAE-cellulose. The resin was then packed into a column and the protein was eluted with the Tris/2-mercaptoethanol buffer containing a linear gradient of NaCl (0–0.5 M). Cytosolic protein fractions eluted at 0.19–0.20 M-NaCl were pooled, batch-absorbed on CM-Sephadex C-50, treated with 0.1 mM-cyclic AMP to release the catalytic subunit from the holoenzyme, and packed into a column. The catalytic subunit was then recovered by elution from the column with a linear gradient of sodium phosphate buffer, pH 6.7 (10–300 mM), and treated once again with CM-Sephadex to remove additional contaminating proteins. Further purification of the catalytic subunit to

electrophoretic homogeneity on polyacrylamide gels containing sodium dodecyl sulphate was accomplished by passage through Sephacryl S-200 as described by Peters *et al.* (1977). Bovine serum albumin (0.5 mg/ml) was added to both enzyme fractions to increase stability, and the preparations were stored at 4°C . Histone and casein kinase activities in the less-purified preparation were completely independent of cyclic AMP or cyclic GMP, and could be eradicated by the specific heat-stable protein inhibitor of the catalytic subunit of cyclic AMP-dependent protein kinase (Ashby & Walsh, 1972). Moreover, phosphoprotein phosphatase activity could not be detected with [^{32}P]phosphohistone as substrate in the method of Mumby & Traugh (1979).

Assay for protein kinase activity

Protein kinase activities in purified cerebral ribosome preparations and in the protein fractions obtained during isolation of the catalytic subunit of the cyclic AMP-dependent enzyme were assayed by a modification of the procedures of Maeno *et al.* (1971) and Corbin & Reimann (1974). The assay mixture contained 5 μmol of sodium acetate, pH 6.5, 1 μmol of NaF, 30 nmol of EGTA, 200 nmol of theophylline, 100 μg of histone II-A or 300 μg of casein, 1 μmol of MgCl_2 , 21.5 nmol of [$\gamma\text{-}^{32}\text{P}$]ATP (50–200 d.p.m./pmol) and, when present, 0.75–1.0 nmol of cyclic AMP or 1.0–5.0 nmol of cyclic GMP in a total volume of 75 μl . The reaction was started by addition of the enzyme preparation (25 μl) and the mixture was incubated at 30°C for 10 min. At the end of the incubation time, 50 μl of assay mixture was transferred to a Whatman 3MM filter-paper disc (23 mm diameter) and the disc was placed in a wire basket suspended in 500 ml of a solution composed of 2.3 mM-silicotungstic acid and 250 mM- H_2SO_4 at 0°C . After at least 10 min, the basket was transferred to 500 ml of 10% (w/v) trichloroacetic acid and heated at 90°C for 15 min. The discs were washed as described by Corbin & Reimann (1974), dried and placed in plastic scintillation vials with 10 ml of 3a70B scintillation 'cocktail'. ^{32}P radioactivity was measured in a liquid-scintillation spectrometer with an efficiency that exceeded 90%.

Protein kinase activity was also measured in the isolated cerebral ribosome/protein kinase systems under the conditions employed for ribosomal protein phosphorylation. The procedure described above was followed except that the assay solution contained 0.25 M-sucrose, 50 mM-Tris/HCl buffer, pH 7.3 at 25°C , 25 mM-KCl, 5 mM- MgCl_2 , 100 μg of histone and 20 nmol of [$\gamma\text{-}^{32}\text{P}$]ATP (200–400 d.p.m./pmol) in 75 μl . Enzyme units of protein kinase activity were defined as pmol of [^{32}P]P_i transferred to protein/min at 30°C .

Incubation of cerebral ribosomes with cerebral protein kinase

Phosphorylation of proteins in cerebral ribosomes was performed at 30°C for 30–120 min in buffer containing 0.25 M-sucrose, 50 mM-Tris/HCl buffer, pH 7.3 at 25°C, 25 mM-KCl, 5 mM-MgCl₂ and 1.1 μmol of [γ -³²P]ATP in 4.15 ml. Concentrations of the catalytic subunit and ribosomal subunits or polyribosomes are indicated in the Results section. Unless otherwise indicated, the reaction was stopped by addition of an ice-cold mixture of 50 mM-Tris/HCl buffer, pH 7.6 at 4°C, containing 100 mM-KCl. The resulting suspensions were cooled on ice for 30 min and then centrifuged at 226 000 g for 20 h at 4°C in a Beckman 50Ti rotor to recover the ribosomes or ribosomal subunits. In experiments with polyribosomes, the pelleted ribosomes were subjected to the dissociation and fractionation procedures described previously to obtain pure 40S or 60S subunits (Roberts & Morelos, 1979). The 40S and 60S subunits from experiments with mixtures of subunits were separated on sucrose density gradients. During incubation, polyribosome disruption averaged less than 10% and no subunit breakdown was detected.

Gel electrophoresis of ribosomal proteins

Isolation of ribosomal proteins from purified cerebral 40S and 60S subunits and two-dimensional electrophoresis of the purified proteins on polyacrylamide gels containing 6 M-urea were performed essentially as described earlier (Roberts & Morelos, 1979). The amounts of ribosomal protein applied to the first-dimensional gels averaged approx. 250 μg. Samples of the dissolved proteins (10–20 μl) were also taken for determination of total protein (Peterson, 1977) and radioactivity. The gel slabs were stained with Coomassie Brilliant Blue, then either dried on blotter paper and subjected to radioautography against Kodak No-Screen X-ray film, or sectioned while wet to obtain individual protein spots for further analysis.

Presumptive molecular weights of phosphorylated ribosomal proteins were determined as follows. Excised sections of the two-dimensional gel slabs were diced and placed in 500 μl of solubilization solution containing 600 mM-urea, 8% (w/v) sucrose, 1% (w/v) sodium dodecyl sulphate and 1% (v/v) 2-mercaptoethanol. The liquid was then removed and dialysed for 3 h against 1 litre of buffer containing 10% (w/v) sucrose, 5 mM-sodium phosphate, pH 7.2, and 0.1% sodium dodecyl sulphate. The dialysis medium was then replaced with fresh buffer containing 30% (w/v) sucrose, instead of 10% sucrose, and dialysis was allowed to proceed overnight. Dialysis was finally continued for an additional 2–4 h in 400 ml of the same buffer containing 40% (w/v) sucrose. The dialysed samples

were then heated in the presence of 5 μl of 2-mercaptoethanol for 15 min at 65°C to dissolve the ribosomal protein. After addition of 0.1% Bromophenol Blue, the protein samples were subjected to electrophoresis in polyacrylamide slab gels containing sodium dodecyl sulphate by a modification of the discontinuous system of Laemmli (1970). The separating gel was composed of 10–15% acrylamide, and the stacking gel contained 5% acrylamide. Electrophoresis was conducted without cooling with a current of 20 mA per slab for 4–5 h or until the tracking dye was within 1 cm from the bottom of the slab. The slab was stained with Coomassie Brilliant Blue.

Materials

Column-chromatography chemicals were obtained from Whatman, Clifton, NJ, U.S.A. (DE-52 DEAE-cellulose), and Pharmacia Fine Chemicals, Piscataway, NJ, U.S.A. (CM-Sephadex C-50 and Sephacryl S-200). Ultrafiltration membranes (PM-30) were obtained from Amicon Corp., Lexington, MA, U.S.A. Purified casein was purchased from Sigma Chemical Co., St. Louis, MO, U.S.A., and subjected to the hydrolysis and partial dephosphorylation procedures described by Ashby & Walsh (1974) before use as a protein kinase substrate. [γ -³²P]ATP was prepared by the method of Post & Sen (1967). All other materials were obtained as described previously (Roberts and Morelos, 1982).

Results

Phosphorylation of ribosomal proteins in isolated cerebral 40S subunits by the catalytic subunit of cyclic AMP-dependent protein kinase

Initial experiments revealed that the identical ribosomal proteins were phosphorylated on exposure of cerebral ribosomes to either the catalytic subunit eluted from CM-Sephadex C-50 columns or the preparation that had subsequently been passed through Sephacryl-200 (Francis, 1980). Since the more-highly purified protein kinase preparation was unstable (Peters *et al.*, 1977; Alhanaty *et al.*, 1979; Ebert & Finn, 1981), and lost considerable activity during prolonged incubation in the phosphorylating system (see also Issinger *et al.*, 1980), subsequent investigations were performed with the partially purified product obtained directly from the Sephadex columns.

Under relatively mild phosphorylation conditions, a large proportion of radioactivity from [γ -³²P]ATP incorporated into ribosomal proteins of the cerebral 40S subunit in the presence of the catalytic subunit of cyclic AMP-dependent protein kinase was concentrated in protein S6 (Fig. 1e). A schematic representation of the protein-stained

Fig. 1. Two-dimensional electrophoresis of ribosomal proteins isolated from cerebral 40S subunits after incubation with the catalytic subunit of cerebral cyclic AMP-dependent protein kinase

Purified cerebral 40S subunits (20 A_{260} units) were incubated in the presence of [γ - 32 P]ATP (930 d.p.m./pmol) and various amounts of the catalytic subunit of cerebral cyclic AMP-dependent protein kinase isolated from CM-Sephadex C-50 columns. Incubation was for 2 h at 30°C in buffer containing 0.25 M-sucrose, 50 mM-Tris/HCl, pH 7.3 at 25°C, 25 mM-KCl and 5 mM-MgCl₂. After incubation, the 40S subunits were recovered by centrifugation at 226 600 g for 18–20 h at 4°C. Protein was prepared from pelleted subunits, analysed for radioactivity, and subjected to two-dimensional polyacrylamide-gel electrophoresis and radioautography. (a), (b), (c) and (d) Electrophoretograms of the two-dimensional gels stained with Coomassie Brilliant Blue. (a) Ribosomal protein from non-incubated 40S subunits. (b) The arrowheads point to the phosphorylated satellite proteins of proteins S2, S3, S7 and S10. (e), (f) and (g) Radioautographs obtained by exposing Kodak No-Screen X-ray film to the electrophoretograms for approx. 15 h. The amounts of catalytic subunit present were (in enzyme units/ A_{260} unit of ribosomes): (b) and (e) 17.6, (c) and (f) 65.0, and (d) and (g) 149. The extent of phosphorylation was (in pmol of [32 P]P_i incorporated/ μ g of ribosomal protein): (b) and (e) 0.75, (c) and (f) 5.2, and (d) and (g) 21.7.

Fig. 2. Schematic diagrams of two-dimensional electrophoretograms of cerebral ribosomal proteins

Ribosomal proteins are labelled according to the nomenclature of McConkey *et al.* (1979), except that the prefixes S and L for small and large ribosomal subunits respectively have been omitted. Identification was aided by determination of apparent molecular weights of the protein-stained spots after elution from the electrophoretograms (see the text). (a) Ribosomal proteins from cerebral 40S subunits; (b) ribosomal proteins from cerebral 60S subunits.

electrophoretograms of cerebral 40S proteins is shown in Fig. 2(a). Several other 40S ribosomal proteins were also labelled under these conditions, including proteins with electrophoretic properties comparable with those of proteins S2, S3a, S7, S10, S14 and S15. Two of these proteins (S14 and S15) do not appear to be phosphorylated in rat cerebral cortex *in vivo* (Roberts & Morelos, 1979), whereas one ribosomal protein phosphorylated *in situ* (S5) incorporated detectable radioactivity from [^{32}P]-ATP only after exposure of isolated cerebral 40S subunits to relatively high activities of the protein kinase (Fig. 1g). The radioactivity associated with a number of the 40S ribosomal proteins co-migrated with small amounts of stained protein that travelled slightly more slowly in the first dimension of two-dimensional gels than did the main body of stained protein (Fig. 1b, arrows). Proteins S6 and S10 were phosphorylated in multiple sites, with the eventual appearance of several radioactive and stained satellites on the electrophoretograms, whereas protein S2 and the other labelled proteins appeared to form only one phosphorylated derivative (see, for example, Figs. 3 and 4). In each case, the congener with greatest mobility was not phosphorylated. Progressive phosphorylation of protein S6 resulted in marked increases in the diphosphorylated derivative of this ribosomal protein (Fig. 3). When the protein-stained and radioactive portions of the two-dimensional gels corresponding to the more-readily phosphorylated 40S ribosomal proteins were eluted separately, each portion travelled with the same electrophoretic mobility on third-dimensional polyacrylamide gels containing sodium dodecyl sulphate. Protein S6 and its phosphorylated congeners all had a mobility corresponding to $M_r = 32\,000$ (not shown). Protein S2 and its

radioactive satellite exhibited a mobility corresponding to approx. $M_r = 33\,000$ (Fig. 5, lanes 1, 2, 7 and 8). Protein S10 and its congeners had a mobility corresponding to $M_r = 16\,700$ (Fig. 5, lanes 5, 6, 11 and 12). Protein S3a (Fig. 5, lane 4, slower component on sodium dodecyl sulphate-containing gels) and its radioactive satellite (Fig. 5, lane 10) had a mobility corresponding to $M_r = 32\,100$, whereas the remaining portion of the S3 complex, which was not radioactive, had a mobility corresponding to $M_r = 29\,700$ (Fig. 5, lanes 3 and 4, faster component). Proteins S5, S7, S14, S15 and their presumptive radioactive analogues had M_r values of approx. 23 000, 22 500, 16 700 and 18 900 respectively (results not shown). These M_r values were similar to those published previously for the analogous ribosomal proteins from rat cerebral cortex (Roberts & Ashby, 1978; Roberts & Morelos, 1979) and rat liver (Lin & Wool, 1974; Collatz *et al.*, 1976, 1977; Tsurugi *et al.*, 1976, 1977, 1978; Lin *et al.*, 1979).

Phosphorylation of ribosomal proteins in isolated 60S subunits by the catalytic subunit of cerebral cyclic AMP-dependent protein kinase

Under the relatively mild phosphorylation conditions that resulted in labelling of a limited number of ribosomal proteins of cerebral 40S subunits, radioactivity from [$\gamma\text{-}^{32}\text{P}$]ATP was incorporated into many ribosomal proteins of the 60S subunit (Fig. 6c). Several of these proteins exhibited electrophoretic mobilities on two-dimensional gels comparable with those of ribosomal proteins previously found to be phosphorylated in rat cerebral cortex *in vivo*, namely proteins L6, L13, L14, L19 and L29 (Roberts & Ashby, 1978; Roberts & Morelos, 1979). The locations of these proteins are shown in

the schematic diagram (Fig. 2b). Subsequent electrophoresis on third-dimensional gels containing sodium dodecyl sulphate revealed that these proteins had apparent M_r values similar to those reported previously for the corresponding ribosomal proteins from rat cerebral cortex (Roberts & Morelos, 1979) and rat liver (Tsurugi *et al.*, 1976, 1977, 1978).

Phosphorylation of ribosomal proteins in mixtures of cerebral ribosomal subunits and polyribosomes by the catalytic subunit of cerebral cyclic AMP-dependent protein kinase

Phosphorylation of ribosomal proteins in cerebral 40S or 60S subunits incubated separately in the presence of the catalytic subunit of cyclic AMP-dependent protein kinase was inversely proportional to the amount of ribosomes present (Table 1, Condition 1 versus 2 and Condition 5 versus 6). The extent of phosphorylation per μg of protein was similar for equal amounts of 40S or 60S subunits (Condition 2 versus 6). Moreover, if equal amounts of subunit protein were present as isolated 40S subunits or as a mixture of 40S and 60S subunits (Condition 2 versus 3), phosphorylation of the 40S subunit was comparable in either case, even though density-gradient analyses revealed considerable formation of monoribosomes in the incubated mixture of ribosomal subunits. In contrast, the 60S subunits in this same mixture were phosphorylated to only about one-third the extent of an equal amount of 60S subunits alone (Condition 6 versus 7). Restriction of ribosomal protein phosphorylation was noted for both subunits when cerebral polyribosomes were incubated with the protein

Fig. 3. Alterations in phosphorylation of the S6 ribosomal protein during incubation of cerebral 40S subunits with the catalytic subunit of cerebral cyclic AMP-dependent protein kinase

Conditions of incubation and separation of ribosomal proteins by two-dimensional gel electrophoresis were similar to those described in the legend to Fig. 1. After the two-dimensional slab gels were stained with Coomassie Brilliant Blue, a rectangular portion of the polyacrylamide gel containing the S6 protein and its phosphorylated satellites was excised, placed in a quartz cell, and scanned for absorbance at 600 nm (—) in a Gilford model 240 spectrophotometer equipped with a linear transport attachment. The excised gel section was then divided into 1 mm cross-sections, and each slice was dissolved in 0.9 ml of 30% (v/v) H_2O_2 by heating for 1 h at 65°C in a glass scintillation vial. To each vial was added 9 ml of scintillation 'cocktail' and radioactivity (—●—) was measured. The amounts of catalytic subunit present were (in enzyme units/ A_{260} unit of ribosomes): (a) 0, (b) 1.1, (c) 17.6 and (d) 149.

Fig. 4. Phosphorylation of ribosomal proteins S2 and S10 during incubation of cerebral 40S subunits with the catalytic subunit of cyclic AMP-dependent protein kinase

Conditions of incubation, separation of ribosomal proteins by two-dimensional gel electrophoresis, and analysis of proteins S2 and S10 and their phosphorylated satellites were similar to those described in the legends to Figs. 1 and 3. (a) and (d), Non-incubated controls. The amounts of catalytic subunit present were (in enzyme units/ A_{260} unit of ribosomes): (b) and (e) 58.6, and (c) and (f) 151.5. —, A_{260} ; —●—, radioactivity.

kinase preparation. This restriction was greater for 60S subunits than for 40S subunits. Thus, compared with the individual isolated subunits, the decrease in ribosomal protein phosphorylation in the polyribosome preparation amounted to about 60% for 40S subunits (Condition 2 versus 4) and about 90% for 60S subunits (Conditions 6 versus 8).

Radioautographs of two-dimensional electro-

phoretograms clearly demonstrated the influence of ribosomal subunit association on the spectrum of cerebral ribosomal proteins phosphorylated by the catalytic subunit of cyclic AMP-dependent protein kinase. Under relatively mild phosphorylation conditions, isolated cerebral 40S subunits incorporated radioactivity from [$\gamma^{32}\text{P}$]ATP into several ribosomal proteins, including proteins S2, S3a, S6, S7,

Fig. 5. Electrophoretic analysis of the phosphorylated congeners of proteins S2, S3 and S10 in polyacrylamide gels containing sodium dodecyl sulphate after incubation of cerebral 40S subunits with the catalytic subunit of cerebral cyclic AMP-dependent protein kinase

Conditions of incubation and separation of ribosomal proteins by two-dimensional electrophoresis were similar to those described in the legend to Fig. 1. Proteins were eluted from selected portions of the two-dimensional gel slabs and subjected to third-dimensional electrophoresis in polyacrylamide gel containing sodium dodecyl sulphate. Lanes 1–6, electrophoretograms stained with Coomassie Brilliant Blue. Lanes 7–12, radioautographs obtained by exposing Kodak No-Screen X-ray film to the electrophoretograms for 1–3 days. Lanes 1 and 7, the satellite of protein S2; lanes 2 and 8, protein S2; lanes 3 and 9, the portion of the S3 complex with slower mobility on second-dimensional gels; lanes 4 and 10, the remaining portion of the S3 complex; lanes 5 and 11, the satellites of protein S10; lanes 6 and 12, protein S10.

Fig. 6. Two-dimensional electrophoresis of ribosomal proteins isolated from cerebral 60S subunits after incubation with the catalytic subunit of cerebral cyclic AMP-dependent protein kinase

Purified 60S subunits ($20 A_{260}$ units) were incubated in the presence of [γ - 32 P]ATP (793 d.p.m./pmol) and the catalytic subunit of cerebral cyclic AMP-dependent protein kinase (1314 enzyme units). The reaction was terminated by the addition of 2 vol. of acetic acid and 0.1 vol. of 1 M-MgCl₂. After dialysis of the incubated suspension for 20 h against 33 vol. of 67% (v/v) acetic acid, rRNA was removed by centrifugation at 900 g for 20 min at 4°C. Ribosomal proteins were subjected to two-dimensional gel electrophoresis. The gel slabs were stained with Coomassie Brilliant Blue, and then placed in contact with Kodak No-Screen X-ray film for 19 h. The electrophoretograms (a) and (b) and radioautographs (c) and (d) were obtained with ribosomal proteins isolated from 60S subunits incubated for 15 min [(a) and (c)] or 60 min [(b) and (d)].

Table 1. *Phosphorylation of ribosomal proteins in separated 40S and 60S subunits, a mixture of subunits, and polyribosomes from rat cerebral cortex in the presence of the catalytic subunit of cerebral cyclic AMP-dependent protein kinase*

Cerebral ribosomal preparations were incubated with the catalytic subunit of cerebral cyclic AMP-dependent protein kinase (2004 enzyme units) in the presence of [γ - 32 P]ATP (995 d.p.m./pmol) for 1 h at 30°C. After incubation, the samples were cooled in ice and then centrifuged at 226 600 g for 20 h at 4°C. Polyribosomes and subunit mixtures were subjected to high-salt dissociation and/or fractionation procedures to obtain purified 40S and 60S subunits (Roberts & Morelos, 1980). Ribosomal proteins were isolated and subjected to two-dimensional electrophoresis. Samples were taken for measurement of protein and radioactivity.

Condition	Ribosomal preparation incubated*	Ribosomal protein (mg)	Ribosomal protein phosphorylation	
			(pmol of [³² P]P _i /μg of protein)	(pmol of [³² P]P _i /pmol of subunit‡)
			40S Subunit	
1	40S	0.51	29.78	23.23
2	40S†	1.51	8.73	6.81
3	40S + 60S	0.51 + 1.01	9.56	7.46
4	Polyribosomes	1.51	3.60	2.81
			60S Subunit	
5	60S	1.01	17.20	23.56
6	60S†	1.52	9.57	13.11
7	40S + 60S	0.51 + 1.01	3.39	4.64
8	Polyribosomes	1.52	0.85	1.16

* The 40S and 60S subunits were present in equimolar concentrations, except where indicated.

† The 40S and 60S subunits in these preparations were present in concentrations such that the total protein was equal to that in the subunit mixtures and polyribosomes.

‡ Based on total molecular weight of 0.78×10^6 and 1.37×10^6 for ribosomal proteins in 40S and 60S subunits respectively. The results shown are representative of replicate experiments.

S10 and S15 (Fig. 7e; see also Fig. 1e). In contrast, incubation of the same quantity of cerebral polyribosomes under the same conditions produced labelling of only proteins S2, S6 and S10 (Fig. 7f). Analysis of the individual congeners of protein S6 revealed that, in common with isolated 40S ribosomal subunits (Fig. 3), exposure of polyribosomes or mixtures of subunits to the catalytic subunit resulted in a marked increase in the incorporation of radioactivity into the diphosphorylated derivative of this protein and smaller increases in phosphorylation of mono-, tri- and tetra-phosphorylated derivatives (results not shown). Association of 60S subunits with 40S subunits in polyribosomes also markedly diminished phosphorylation of cerebral 60S ribosomal proteins. Whereas the isolated 60S subunits incorporated radioactivity into a large number of basic proteins under these conditions (Fig. 7g), only proteins L6, L13, L24, L28 and L29 were reasonably well labelled in incubated polyribosomes (Fig. 7h).

Discussion

These investigations demonstrate that the nature and extent of ribosomal protein phosphorylations produced in isolated ribosome preparations from rat cerebral cortex are markedly dependent on the state of ribosomal subunit association. Under appropriate conditions, the nature and extent of the

ribosomal protein phosphorylations observed in the presence of purified preparations of the catalytic subunit of cerebral cyclic AMP-dependent protein kinase closely resembled the spectrum of phosphorylations described in rat cerebral cortex *in vivo* (Roberts & Ashby, 1978; Roberts & Morelos, 1979). However, these results cannot be interpreted to mean that cyclic AMP-dependent protein kinase is responsible for all of the ribosomal protein phosphorylations observed in rat cerebral cortex *in vivo*. Conditions other than subunit association, such as subcellular compartmentation and relative enzyme–substrate affinity, may restrict the interaction of this and other protein kinases with specific ribosomal protein phosphorylation sites *in vivo*.

In common with other studies of eukaryotic cellular and cell-free preparations (Wool, 1979; Traugh, 1981), protein S6 was the most readily phosphorylated ribosomal protein in reconstituted systems containing cerebral 40S subunits or polyribosomes and the catalytic subunit of cerebral cyclic AMP-dependent protein kinase. Although all of the phosphorylated congeners of protein S6 were produced under these conditions, formation of the more highly phosphorylated species was not as extensive as that observed *in vivo*. Several possible explanations for this difference may be suggested. Thus the findings that protein S6 is normally more highly phosphorylated in polyribosomes than in

Fig. 7. Two-dimensional electrophoresis of 40S and 60S ribosomal proteins after incubation of isolated ribosomal subunits or polyribosomes from rat cerebral cortex in the presence of the catalytic subunit of cerebral cyclic AMP-dependent protein kinase

Cerebral ribosomal preparations (3.57 mg of protein) were incubated with the catalytic subunit of cerebral cyclic AMP-dependent protein kinase (2500 enzyme units) in the presence of [γ - 32 P]ATP (940 d.p.m./pmol) for 1 h at 30°C. (a) (b), (c) and (d), Electrophoretograms stained with Coomassie Brilliant Blue. (e), (f), (g) and (h), Radioautographs obtained by exposing Kodak No-Screen X-ray film to the electrophoretograms for 17–18 h. (a) and (e), 40S subunits incubated alone; (b) and (f), 40S subunits from incubated polyribosomes; (c) and (g), 60S subunits incubated alone; (d) and (h), 60S subunits from incubated polyribosomes. The amounts of phosphate incorporated into ribosomal proteins of the subunits were (in pmol/ μ g of protein): (a) and (e) 1.98, (b) and (f) 0.53, (c) and (g) 3.28, and (d) and (h) 0.18.

monoribosomes or subunits (Leader & Coia, 1978; Roberts & Morelos, 1980), and that phosphorylation of this protein is associated with the initiation of protein synthesis (Martini & Kruppa, 1979; Wettenhall & Howlett, 1979; Thomas *et al.*, 1980), indicate that protein-S6 phosphorylation may be maximal *in vivo* during polyribosome formation. In addition, protein kinases other than the cyclic AMP-dependent enzyme may normally be responsible for phosphorylating certain sites on protein S6 (Floyd & Traugh, 1981; Lastick & McConkey, 1981).

Phosphorylation of several 40S ribosomal proteins in addition to S6 protein has been described in eukaryotic cells *in vivo* (for a review see Traugh, 1981). In rat cerebral cortex, these include proteins S2, S3a and S5 and a radioactive protein that trailed protein S10 in the first dimension on two-dimensional electrophoretograms (Roberts & Ashby, 1978; Roberts & Morelos, 1979). These cerebral ribosomal proteins were also phosphorylated in isolated cerebral 40S subunits in the presence of the catalytic subunit of cyclic AMP-dependent protein kinase from the same tissue. The extent of phosphorylation of protein S10 was second only to that of protein S6. Moreover, in common with protein S6, protein S10 exhibited multiple sites of phosphorylation. In contrast, protein S10 was phosphorylated to a much smaller extent in 40S subunits from HeLa cells and Krebs II ascites-tumour cells in the presence of the catalytic subunit of cyclic AMP-dependent protein kinase from bovine cardiac muscle (Issinger *et al.*, 1980). In the latter studies, protein S10 was more rapidly phosphorylated by cyclic GMP-dependent protein kinase from the same source. However, in view of the fact that protein S10 serves as a preferential substrate for a proteinase-activated protein kinase in isolated 40S ribosomal subunits from rabbit reticulocytes (Del Grande & Traugh, 1979), the protein kinase responsible for phosphorylation of this ribosomal protein *in vivo* remains obscure.

Phosphorylation of a number of basic proteins of 60S ribosomal subunits in eukaryotic cells *in vivo* has also been reported. These include proteins L6, L13, L14, L19 and L29 in rat cerebral cortex *in vivo* (Roberts & Ashby, 1978; Roberts & Morelos, 1979). In the present investigations with isolated cerebral 60S subunits in the presence of the catalytic subunit of cyclic AMP-dependent protein kinase from rat cerebral cortex, radioactivity from [γ - 32 P]ATP was readily incorporated into proteins L6, L13 and L14 and less rapidly into proteins L19 and L29. Overall phosphorylation of ribosomal proteins was more rapid in isolated cerebral 60S subunits than in the corresponding 40S subunits (see also Eil & Wool, 1973a; Walton & Gill, 1973; Issinger *et al.*, 1980). It is noteworthy that 60S ribosomal proteins were also phosphorylated to a greater extent than

40S ribosomal proteins during incubation of rat cerebral cells *in vitro* (Roberts & Ashby, 1978). The reverse situation has been observed in rat cerebral cortex *in situ* (Roberts & Morelos, 1979).

In the present experiments, the availability or reactivity of many ribosomal protein phosphorylation sites was severely limited in cerebral polyribosomes compared with monoribosomes or ribosomal subunits. This phenomenon has also been noted by Cenatiempo *et al.* (1977), who found that only proteins S6 and L18 were phosphorylated in free polyribosomes from rat liver incubated with a commercial preparation of protein kinase from bovine heart. In the present studies, the restriction was more pronounced for 60S subunits, with the result that the relative ability of certain 40S ribosomal proteins to function as substrates for the catalytic subunit of cyclic AMP-dependent protein kinase increased under these conditions. Ribosomal proteins that were phosphorylated in isolated cerebral free polyribosomes by the catalytic subunit of cerebral cyclic AMP-dependent protein kinase included proteins S2, S6 and S10 of the 40S subunit, as well as proteins L6, L13 and L29 of the 60S subunit. However, other cerebral ribosomal proteins that were also phosphorylated *in vivo* did not incorporate significant amounts of radioactivity in isolated polyribosomes, e.g. proteins S3a, S5, L14 and L19. Since these proteins were phosphorylated only in ribosomal subunits in the isolated systems, their phosphorylation may be restricted to ribosomal subunits *in situ* as well.

The functional consequences that result from phosphorylation of specific structural ribosomal proteins are unknown. Cross-linking studies indicate that proteins S3 and S6 may function in mRNA binding (Terao & Ogata, 1979a,b; Stahl & Kobets, 1981), and protein S3 can bind the initiation factor eIF-2 and tRNA^{fMet}, which are components of the quaternary initiation complex with 40S ribosomal subunits (Westermann *et al.*, 1979, 1981). Proteins L6 and L19 are prominent among the ribosomal proteins that bind strongly to 5S and 5.8S rRNA (Todokoro *et al.*, 1981). Protein L6 has been observed to bind strongly to tRNA as well (Ulbrich *et al.*, 1980). However, conflicting results have been obtained on the possible relationship between ribosomal protein phosphorylation and protein synthesis in eukaryotic cells (Eil & Wool, 1973b; Grankowski *et al.*, 1979; Wettenhall & Howlett, 1979; Thomas *et al.*, 1980; Leader *et al.*, 1981; Mastropaolo & Henshaw, 1981; Nielsen *et al.*, 1981; Roberts, 1982). The availability of isolated ribosome-protein kinase systems capable of carrying out relatively specific and restricted ribosomal protein phosphorylations that resemble the phosphorylations observed *in vivo* should facilitate resolution of this problem.

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References

- Alhanaty, E., Bashan, N., Moses, S. & Shaltiel, S. (1979) *Eur. J. Biochem.* **101**, 283–290
- Ashby, C. D. & Walsh, D. A. (1972) *J. Biol. Chem.* **247**, 6637–6642
- Ashby, C. D. & Walsh, D. A. (1974) *Methods Enzymol.* **38C**, 350–358
- Bechtel, P. J., Beavo, J. A. & Krebs, E. G. (1977) *J. Biol. Chem.* **252**, 2691–2697
- Cenatiempo, Y., Cozzzone, A. J., Genot, A. & Reboud, J. P. (1977) *FEBS Lett.* **79**, 165–169
- Collatz, E., Wool, I. G., Lin, A. & Stöffler, G. (1976) *J. Biol. Chem.* **251**, 4666–4672
- Collatz, E., Ulbrich, N., Tsurugi, K., Lightfoot, H. N., MacKinlay, W., Lin, A. & Wool, I. G. (1977) *J. Biol. Chem.* **252**, 9071–9080
- Corbin, J. D. & Reimann, E. M. (1974) *Methods Enzymol.* **38C**, 287–290
- Del Grande, R. & Traugh, J. A. (1979) *J. Supramol. Struct. Suppl.* **3**, 28
- Ebert, R. F. & Finn, F. M. (1981) *Endocrinology* **109**, 197–204
- Eil, C. & Wool, I. G. (1973a) *J. Biol. Chem.* **248**, 5122–5129
- Eil, C. & Wool, I. G. (1973b) *J. Biol. Chem.* **248**, 5130–5136
- Floyd, G. A. & Traugh, J. A. (1981) *Eur. J. Biochem.* **117**, 257–262
- Francis, T. A. (1980) Ph.D. Thesis, University of California at Los Angeles
- Francis, T. A. & Roberts, S. (1978) *Fed. Proc. Fed. Am. Soc. Exp. Biol.* **37**, 1406
- Francis, T. A. & Roberts, S. (1980) *Fed. Proc. Fed. Am. Soc. Exp. Biol.* **39**, 1743
- Grankowski, N., Kramer, G. & Hardesty, B. (1979) *Arch. Biochem. Biophys.* **197**, 618–629
- Issinger, O.-G., Beier, H., Speichermann, N., Flokerzi, V. & Hofmann, F. (1980) *Biochem. J.* **185**, 89–99
- Laemmli, U. K. (1970) *Nature (London)* **227**, 680–685
- Lastick, S. M. & McConkey, E. H. (1981) *J. Biol. Chem.* **256**, 583–585
- Leader, D. P. (1980) in *Recently Discovered Systems of Enzyme Regulation by Reversible Phosphorylation* (Cohen, P., ed.), pp. 203–233, Elsevier/North-Holland, Amsterdam
- Leader, D. P. & Coia, A. A. (1978) *FEBS Lett.* **90**, 270–274
- Leader, D. P., Thomas, A. & Voorma, H. O. (1981) *Biochim. Biophys. Acta* **656**, 69–75
- Lin, A. & Wool, I. G. (1974) *Mol. Gen. Genet.* **134**, 1–6
- Lin, A., Tanaka, T. & Wool, I. G. (1979) *Biochemistry* **18**, 1634–1637
- Maeno, H., Johnson, E. M. & Greengard, P. (1971) *J. Biol. Chem.* **246**, 134–142
- Martini, O. H. W. & Kruppa, J. (1979) *Eur. J. Biochem.* **95**, 349–358
- Mastropaolo, W. & Henshaw, E. C. (1981) *Biochim. Biophys. Acta* **656**, 246–255
- McConkey, E. H., Bielka, H., Gordon, J., Lastick, S. M., Lin, A., Ogata, K., Reboud, J.-P., Traugh, J. A., Traut, R. R., Warner, J. R., Welfle, H. & Wool, I. G. (1979) *Mol. Gen. Genet.* **169**, 1–6
- Mumby, M. & Traugh, J. A. (1979) *Biochemistry* **18**, 4548–4556
- Nielsen, P. J., Duncan, R. & McConkey, E. H. (1981) *Eur. J. Biochem.* **120**, 523–527
- Peters, K. A., Demaille, J. G. & Fischer, E. H. (1977) *Biochemistry* **16**, 5691–5697
- Peterson, G. L. (1977) *Anal. Biochem.* **83**, 346–356
- Post, R. L. & Sen, A. K. (1967) *Methods Enzymol.* **10**, 773–776
- Roberts, S. (1982) *Characterization and Function of Brain Phosphoproteins: Prog. Brain Res.* **56** in the press
- Roberts, S. & Ashby, C. D. (1978) *J. Biol. Chem.* **253**, 288–296
- Roberts, S. & Morelos, B. S. (1979) *Biochem. J.* **184**, 233–244
- Roberts, S. & Morelos, B. S. (1980) *Biochem. J.* **190**, 405–419
- Roberts, S. & Morelos, B. S. (1982) *Biochem. J.* **202**, 343–351
- Stahl, J. & Kobets, N. D. (1981) *FEBS Lett.* **123**, 269–272
- Terao, K. & Ogata, K. (1979a) *J. Biochem. (Tokyo)* **86**, 597–603
- Terao, K. & Ogata, K. (1979b) *J. Biochem. (Tokyo)* **86**, 605–617
- Thomas, G., Siegmann, M., Kubler, A.-M., Gordon, J. & Jimenez de Asua, L. (1980) *Cell* **19**, 1015–1023
- Todokoro, K., Ulbrich, N., Chan, Y.-L. & Wool, I. G. (1981) *J. Biol. Chem.* **256**, 7207–7212
- Traugh, J. A. (1981) *Biochem. Actions Horm.* **8**, 167–208
- Tsurugi, K., Collatz, E., Wool, I. G. & Lin, A. (1976) *J. Biol. Chem.* **251**, 7940–7946
- Tsurugi, K., Collatz, E., Todokoro, K. & Wool, I. G. (1977) *J. Biol. Chem.* **252**, 3961–3969
- Tsurugi, K., Collatz, E., Todokoro, K., Ulbrich, N., Lightfoot, H. N. & Wool, I. G. (1978) *J. Biol. Chem.* **253**, 946–955
- Ulbrich, N., Wool, I. G., Ackerman, E. & Sigler, P. B. (1980) *J. Biol. Chem.* **255**, 7010–7016
- Ventimiglia, F. A. & Wool, I. G. (1974) *Proc. Natl. Acad. Sci. U.S.A.* **71**, 350–354
- Walton, G. M. & Gill, G. N. (1973) *Biochemistry* **12**, 2604–2611
- Westermann, P., Heumann, W., Bommer, U.-A., Bielka, H., Nygård, O. & Hultin, T. (1979) *FEBS Lett.* **97**, 101–104
- Westermann, P., Nygård, O. & Bielka, H. (1981) *Nucleic Acids Res.* **9**, 2387–2396
- Wettenhall, R. E. H. & Howlett, G. J. (1979) *J. Biol. Chem.* **254**, 9317–9323
- Wool, I. G. (1979) *Annu. Rev. Biochem.* **48**, 719–754