Phosphorylation of Ribosomal Proteins of *Escherichia coli* by Protein Kinase from Rabbit Skeletal Muscle†

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ABSTRACT: Specific proteins from the 30S and 50S ribosomal subunits of *Escherichia coli* MRE 600 as well as proteins removed from the ribsome by 0.5 M NH_4Cl accept phosphate from [γ-^{32}P]ATP in a reaction catalyzed by the catalytic subunit of cAMP-dependent protein kinase from rabbit skeletal muscle. Phosphorylation occurs at both serine and threonine residues. The four receptor proteins in the 30S subunit have been identified by polyacrylamide gel electrophoresis in urea and dodecyl sulfate. These proteins have been identified as 9 (S4), 5 (S9), 2b (S18), and 2a (S19). Protein 5, the major phosphorylated species in the 30S subunit, accepts up to 0.3 mole of phosphorus/mole of protein. The identity of the proteins has been confirmed by phosphorylation of the individual pure 30S proteins. The specificity of phosphorylation of the 30S proteins is not dependent upon the configuration of the ribosome since the same proteins are phosphorylated in the native ribosome, the mixed total proteins and pure proteins tested individually. However, the relative amounts of phosphate incorporated by the four proteins in the intact ribosome are different than in the extracted proteins. At least seven proteins of the 50S subunit are phosphorylated. The major receptor protein is 13–14 X (L2). Kinetic data show this protein is the most rapidly labelled species in the 70S ribosome. The other proteins have been identified as 15 IV, 13–14 III (L5), and/or 12 III (L3), 7 VII, 5 VI, 1 IX (L33), and/or 1 XI and 11 X. The relative labeling of the proteins is different in the intact subunit and the extracted proteins.

Proteins associated with mammalian ribosomes from several sources have been shown to become phosphorylated both *in vivo* from ^{32}P (Loeb and Blat, 1970; Kabat, 1970; Walton *et al.*, 1971) and *in vitro* from [γ-^{32}P]ATP (Loeb and Blat, 1970; Kabat, 1971; Eil and Wool, 1971). The biological significance of this phosphorylation is still unclear; however, the regulation by cAMP[1]-dependent protein kinases of other metabolic pathways in mammalian systems (Krebs, 1972) suggests the possibility that protein synthesis might be controlled by means of phosphorylation of specific ribosomal proteins.

Detailed information concerning the chemistry and function of the individual ribosomal proteins of *Escherichia coli* exists, in particular, for those from the 30S subunit. Comparable data for ribosomal proteins from mammalian sources is lacking. On the other hand, well-characterized purified protein kinases have been obtained from mammalian tissues (Krebs, 1972) but not from procaryotes. Accordingly, a hybrid system consisting of ribosomes or ribosomal proteins from *E. coli* and the catalytic subunit of a cAMP-dependent protein kinase from rabbit skeletal muscle has been employed to investigate phosphorylation of ribosomal proteins.

The results presented here show a high degree of substrate specificity in the phosphorylation of ribosomal proteins; a limited number of proteins from both 50S and 30S subunits are phosphorylated. The specificity appears to be due more to the primary structures of the receptor proteins than to steric effects caused by the topographic position of the proteins in the ribosome structure.

Materials

Purified Ribosomal Subunits. E. coli strain MRE 600 was grown in rich medium and harvested during exponential growth at an optical density at 540 nm of 5.0 (Moore *et al.*, 1968). The frozen cell paste was extracted by grinding with alumina in 0.01 Tris (pH 7.4), 0.01 M $MgCl_2$, 0.1 M NH_4Cl, and 0.5 mM dithiothreitol (buffer A). Ribosomes were recovered from the extract by centrifugation in a Spinco 30 rotor at 30,000 rpm for 17 hr and resuspended in buffer A, and the solution was centrifuged at 12,000*g* for 10 min. The ribosome suspension (10 ml) was layered over 30 ml of 30% sucrose, 0.01 M Tris-HCl (pH 7.4), 0.5 M NH_4Cl, and 0.03 M MgCl, and centrifuged 21 hr at 40,000 in a Spinco Ti 60 rotor. The upper two-thirds of the contents of the tube was carefully removed, concentrated to one-fourth the original sample volume with Sephadex G-25 and dialyzed against 0.01 M Tris-HCl (pH 7.4), 0.1 M NH_4Cl, 0.001 M $MgCl_2$, and 0.5 mM dithiothreitol (buffer B). This fraction contained the proteins extracted from ribosomes by 0.5 M NH_4Cl. The ribosome pellets were resuspended in buffer A, placed at −70° for 1 hr, thawed, and then centrifuged for 30 min at 30,000*g* in order to remove yellow material present in impure ribosome preparations. The ribosomes were dissociated into subunits by dialysis against buffer B, and the subunits were separated by zonal centrifugation (Eikenberry *et al.*, 1969).

Ribosomal Protein. Total ribosomal proteins were prepared by digesting the 30S or 50S rRNA with 1 μg of pancreatic RNase/mg of subunit in 4 M urea and 0.01 M EDTA for 30 min at 30°. Then solid urea was added to saturation at 5° to solubilize precipitated protein and the solution was dialyzed against 0.01 M Tris-HCl (pH 7.4), 0.2 M NH_4Cl at 4° for 16 hr.

Purified Ribosomal Proteins. Purified proteins of the 30S ribosomal subunit were prepared by T. A. Bickle in the laboratory of A. Tissieres in Geneva, Switzerland.

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[1] Abbreviation used is: cAMP, adenosine 3′,5′-monophosphate.

TABLE I: Phosphorylation of *E. coli* Ribosomal Protein.[a]

Substrate	pmoles of ^{32}P/ pmole of Subunit
30S Subunit	0.4
Minus protein kinase	0.0
Total 30S protein	1.1
50S Subunit	0.6
Minus protein kinase	0.0
Total 50S protein	1.1
None	0.0

[a] The reaction conditions, described in Methods, were optimized for maximal incorporation of phosphorus into trichloroacetic acid precipitable material.

Protein Kinase. Preparations of the catalytic subunit of cAMP-dependent protein kinase from rabbit skeletal muscle (Reimann *et al.*, 1971a) were a gift of J. D. Corbin, C. O. Brostrom, and E. G. Krebs. The catalytic subunit does not require cAMP for activity.

[γ-^{32}P]ATP. [γ-^{32}P]ATP (specific activity, approximately 1 Ci/mmole) was prepared by a modification of the methods of Glynn and Chappell (1964) as described previously (Reimann *et al.*, 1971b).

Methods

Polyacrylamide Gel Electrophoresis. Ribosomal proteins were analyzed by polyacrylamide gel electrophoresis in two analytical systems, either in gels containing 8 M urea at pH 4.5, or sodium dodecyl sulfate. Details of the methods have been published (Traut *et al.*, 1969; and references therein).

Phosphorylation of Ribosomal Subunits or Ribosomal Proteins. The reaction volume of 0.07 ml contained: 14 mM sodium phosphate (pH 7.2), 2 mM magnesium chloride, 0.2 mM [γ-^{32}P]ATP, approximately 0.5 μg of protein kinase, and 5–15 μg of 50S or 30S ribosomal subunits, or the equivalent amount of total protein from each subunit. The reaction conditions for ribosomal protein differed from those above by the inclusion of 0.2 M NH_4Cl to increase solubility of the proteins. The reaction was initiated by adding protein kinase and continued at 30° for 25 min. Samples of 0.05 ml were pipetted onto 2-cm squares of Whatman ET 31 filter paper which were placed in cold 10% trichloroacetic acid, dried, and counted in scintillation fluid (Reimann *et al.*, 1971a).

Autoradiography. Polyacrylamide gels were strained with Coomassie Brilliant Blue, photographed, and then sliced longitudinally. The slices were placed on Whatman No. 3-MM chromatography paper and dried under vacuum (Fairbanks *et al.*, 1965). The dried gel slices were autoradiographed on Kodak No-Screen Medical X-Ray film for 1–3 days. The autoradiograms were scanned at 600 nm with the scanning attachment of the Gilford 2400 spectrophotometer.

Analysis of Serine Phosphate and Threonine Phosphate. The methods used were modifications of those described by Langan (1968). Proteins were partially hydrolyzed in 2 N HCl for 16 hr at 105° in a vacuum-sealed tube. The HCl was removed under vacuum and the residues were resuspended in H_2O and dried under vacuum two additional times. The final residue was dissolved in H_2O and samples were applied to cellulose-coated thin-layer chromatography plates (E. Merck, Darmstradt). Electrophoresis was carried out in formic–acetic acid buffer pH 1.9 for 1.5 hr at 500 V. Serine and threonine phosphate were detected by staining with ninhydrin and identified by comparison to serine and threonine phosphate standards (Sigma Chemical Co.).

Quantitation of Phosphate Incorporation. The total amount of phosphate incorporated into ribosomal particles or the protein derived therefrom was determined from the amount of trichloroacetic acid precipitable radioactive material, the specific activity of the [^{32}P]ATP, and the amount of ribosomes assuming $\epsilon_{260}^{1\%} = 150$. The molar amount of phosphate incorporated into pure 30S proteins was calculated using published molecular weights (Traut *et al.*, 1969) and the amount of protein estimated from the gels stained with Coomassie Brilliant Blue (Bickle and Traut, 1971).

Results

Phosphorylation of Ribosomal Subunits and Extracted Ribosomal Protein. The incorporation of radioactive phosphate from [γ-^{32}P]ATP into the 30S and 50S ribosomal subunits is shown in Table I. Approximately 0.5 mole of phosphate was incorporated into both the 30S and 50S particles, and roughly twice as much into the total protein extracted from each particle. The reaction was completely dependent on the presence of the catalytic subunit of the rabbit skeletal muscle protein kinase; ribosomes alone did not incorporate phosphate. Total ribosomal protein prepared by digestion of ribosomes with RNase incorporated more phosphate than did the equivalent amount of protein contained in the intact ribosomal subunits. Reaction conditions were optimized for maximal incorporation of phosphorus into trichloroacetic acid precipitated material. The extent of phosphorylation was not increased by longer incubation time nor by addition of more ATP or protein kinase. The relative rate of phosphorylation of 30S ribosomal proteins from *E. coli* using rabbit skeletal muscle protein kinase was determined by comparison to casein and histone II-A. The intact 30S subunit incorporated 0.3 pmole/min per μg of protein kinase under saturating substrate concentrations. This was 10-fold less than the rate of phosphate incorporation with casein and 40-fold less than histone II-A.

Phosphorylation of Proteins Removed from Ribosomes by 0.5 M NH_4Cl. Proteins associated with the ribosomes and removed from crude 70S preparations by 0.5 M NH_4Cl were found to incorporate phosphate when incubated with [γ-^{32}P]ATP and catalytic subunit. This protein fraction was analyzed on dodecyl sulfate gels after phosphorylation as shown in Figure 1, and several discrete radioactive bands were present. The molecular weight of the phosphorylated components in the fraction removed from the ribosome by ammonium chloride are >100,000, 97,000, 77,000, 56,000, 32,000, and 11,000. With the exception of the component at 11,000, they do not correspond to the phosphorylated proteins found on 50S and 30S subunits (see below).

Identification of ^{32}P-Labeled Ribosomal Proteins. The phosphorylated proteins from both ribosomal subunits were analyzed by polyacrylamide gel electrophoresis in dodecyl sulfate and at pH 4.5 in urea followed by radioautography and comparison of the radioautograms to the stained protein band patterns. The dodecyl sulfate and urea gels and their corresponding radioautograms of the phosphorylated 30S proteins are shown in Figure 2, and those of the 50S proteins

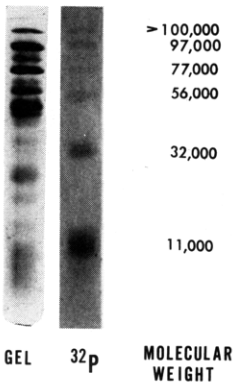

FIGURE 1: Phosphorylated proteins eluted from ribosomes by 0.5 M NH_4Cl. The pattern of proteins present in the 0.5 M NH_4Cl ribosomal wash after phosphorylation and electrophoresis in 10% dodecyl sulfate acrylamide gels is shown on the left after staining with Coomassie Brilliant Blue. The radioactive protein bands are shown on the right as visualized by radioautography of a single gel slice. The corresponding molecular weight of each band was calculated from appropriate protein standards (Traut *et al.*, 1969).

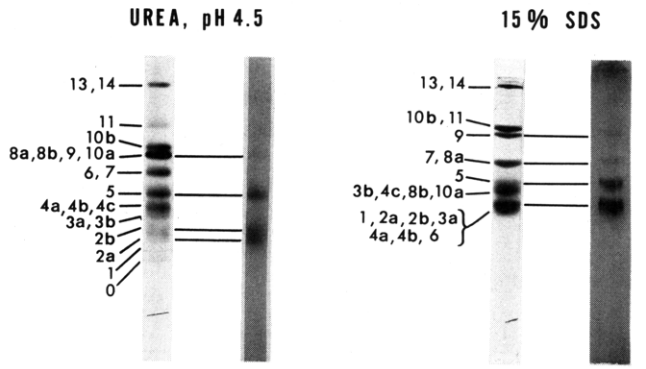

FIGURE 2: Analysis by gel electrophoresis of phosphorylated tota protein from the 30S subunit. Total protein was extracted from the 30S subunit, phosphorylated, and analyzed by gel electrophoresis at pH 4.5 with urea and 15% dodecyl sulfate as described in Methods. The protein pattern stained with Coomassie Brilliant Blue is on the left in each system and the corresponding radioautogram is on the right. The individual protein species have been identified previously (Traut *et al.*, 1969; Dzionara *et al.*, 1970; Wittmann *et al.*, 1971).

are shown in Figure 3. Positive identification of a protein was based on its behavior in the two analytical systems. For both subunits, phosphorylation is highly specific: only a few discrete labeled bands were evident and these coincided with stained protein bands.

The information obtained from the two different gel systems indicates the labeled material in the 30S subunit (Figure 2) consists of proteins 9 (S4), 5 (S9), 2b (S18), and 2a (S19) (Traut *et al.*, 1969; Wittmann *et al.*, 1971), and those in the 50S subunit (Figure 3) consist of 15 IV, mol wt 28,000; 13–14 X (L2), mol wt 36,000; 13–14 III (L5) and/or 12 III (L3), mol wt 24,000; 7 VII, mol wt 15,000; 5 VI, mol wt 11,000; 1 XI (L33) and/or 1 IX, mol wt 9000; and 11 X (Traut *et al*, 1969; Wittman *et al.*, 1971; P. Pearson, H. Delius, and R. R. Traut, unpublished results). Protein 5 (S9) of the 30S subunit and 13–14 X (L2) of the 50S subunit are the predominant phosphate receptors in the intact subunits. The kinetics of phosphorylation show protein 13–14 X (L2) of the 50S subunit is the most rapidly phosphorylated protein when both subunits are present.

The proteins in the intact 30S subunit and the total protein extracted from the subunit were phosphorylated and analyzed, and the patterns of phosphorylation compared. The results are shown in Figure 4. The same 30S proteins become phosphorylated either in the intact particle or in the extracted proteins; however, the relative amount of phosphate incorporated into the different proteins is altered. The most rapidly migrating band which contains proteins 2a and 2b is phosphorylated to a greater degree in the extracted proteins than in the native 30S particle. In the 50S subunit (Figure 3) several additional protein bands are phosphorylated when the proteins are extracted, and the extent of phosphorylation of many of the other proteins is altered.

Phosphorylation of Pure 30S Ribosomal Proteins. The identification of the phosphorylated 30S proteins was confirmed by testing single pure 30S proteins as substrates. Of the 21 30S proteins, all except proteins 0, 1, 2b, 3b, and 10a were assayed. Table II gives the phosphate incorporation of the pure proteins measured by trichloroacetic acid precipitability of ^{32}P. The three pure proteins which incorporated the highest amount of phosphate are among the four found to be phosphorylated in the ribosome and total protein (the fourth protein, 2b, was not available). Four additional pure proteins incorporated a smaller amount of phosphate; these were not routinely detected in the experiments with ribosomes and total protein. The majority of pure 30S proteins do not incorporate phosphate. Purified ribosomal proteins incorporated fewer moles of phosphate per mole of protein than the proteins present in the total ribosome. This may be due to partial denaturation of the individual proteins during purification.

The phosphorylated pure proteins 9 (S4), 5 (S9), and 2a (S19) were analyzed by gel electrophoresis in urea at pH 4.5 followed by radioautography. The results shown in Figure 5 show that the radioactive material coincides with the stained protein band which corresponds to the pure protein.

The purity of the ribosomal proteins had been established by their behavior in two analytical gel systems. It is highly unlikely that minor contaminants with the same electrophoretic properties as ribosomal proteins might be respon-

TABLE II: Phosphorylation of Purified 30S Proteins.[a]

Major ^{32}P Acceptors		Minor ^{32}P Acceptors		Nonacceptors	
Protein	pmole of ^{32}P/pmole of Protein	Protein	pmole of ^{32}P/pmole of Protein	Protein	pmole of ^{32}P/pmole of Protein
2_a	0.06	3_a	0.01		
5	0.16	7	0.03	4_a	0
9	0.04	8_b	0.02	4_b	0
		10_b	0.02	4_c	<0.01
				6	<0.01
				8_a	<0.01
				10_{a2}	0
				10_{a3}	0
				11	0
				13	0

[a] Not Shown: $0, 1, 2_b, 3_b, 10_{a1}$.

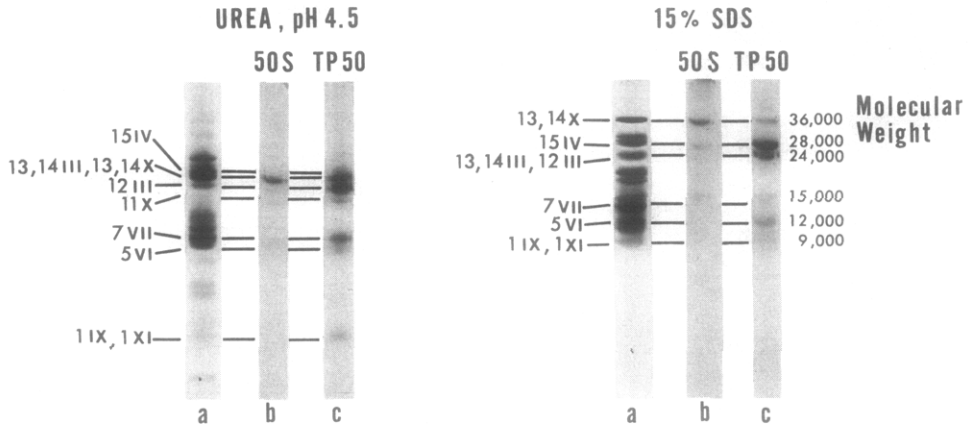

FIGURE 3: Analysis by gel electrophoresis of phosphorylated total protein from the 50S subunit. Total protein from the 50S subunit was phosphorylated and analyzed by gel electrophoresis at pH 4.5 with urea and 15% dodecyl sulfate as described in Methods. The protein pattern is on the left in each system (a and d). The corresponding radioautograms of the intact 50S subunit (b and e) and of the total protein extracted from the dodecyl sulfate subunit (c and f) are shown. The individual protein species have been identified previously (Traut *et al.*, 1969).

sible for phosphate acceptor activity instead of the ribosomal proteins themselves.

Characterization of the Site of Phosphorylation. Partial acid hydrolysates of both the total phosphorylated proteins from 50S and 30S subunits and of the three phosphorylated pure 30S proteins were analyzed by thin-layer electrophoresis at pH 1.9 in order to identify the site of phosphate incorporation. The results in Figure 6 show that both serine phosphate and threonine phosphate occur in the total proteins of both subunits, and that serine is the major phosphate acceptor. Serine phosphate was found in the pure 30S protein 5 (S9); threonine was the predominant phosphate acceptor in proteins 9 (S4) and 2a (S19) although smaller amounts of serine phosphate were present. (The hydrolysate from protein P5 was underexposed.) Since protein 5 (S9) is the major 30S phosphate receptor, serine phosphate is the major phosphorylated amino acid in the total 30S protein.

Effect of Phosphorylation on Activity. Phosphorylation of the 70S ribosomes under conditions in which each ribosome contained approximately 0.5 mole of phosphate led to a 25% loss of activity in protein synthesis in a DNA directed system (Table III). Under these conditions the 50S protein 13–14 X (L2) is the major phosphate acceptor and the results suggest that the inactivation may reside primarily in the 50S subunit.

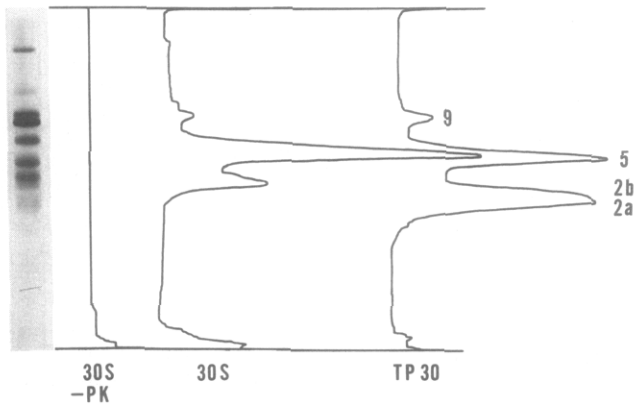

FIGURE 4: Phosphorylation of 30S ribosomes and total protein extracted from the 30S subunit. The pattern of proteins from the 30S subunit after electrophoresis in urea, pH 4.5, and staining with Coomassie Brilliant Blue is shown at the left of the figure. Spectrophotometric scans of radioautograms of dried gel slices include: 30S subunit incubated in the absence of protein kinase from rabbit skeletal muscle (30S − PK); 30S subunit incubated in the presence of protein kinase (30S); total protein extracted from the 30S subunit and incubated in the presence of protein kinase (TP 30).

Discussion

Three types of ribosomal substrates were found to have acceptor activity for the transfer of [^{32}P]phosphate from [γ-^{32}P]ATP catalyzed by skeletal muscle protein kinase: intact *E. coli* ribosomal subunits, ribosomes digested with ribonuclease, and purified individual ribosomal proteins. Coelectrophoresis of the labeled material with major ribosomal protein components in several gel electrophoretic systems

TABLE III: Activity of Phosphorylated Ribosomes.[a]

	T4-DNA	pmoles of Leucine/ pmole of Ribosome	pmoles of ^{32}P/pmole of Ribosome	% Inhibn
Ribosomes	+	1.25	0.03	
Phosphorylated ribosomes	+	0.94	0.52	25
Ribosomes	−	0.50	0.03	
Phosphorylated ribosomes	−	0.31	0.54	38

[a] Ribosomes (0.6 mg) were incubated with [γ-^{32}P]ATP in the presence and absence of protein kinase as described in Methods, then dialyzed against buffer C. Reaction mixtures for T4-DNA-dependent protein synthesis contained 50 mM Tris-acetate (pH 8.0), 100 mM NH_4Cl, 50 mM potassium acetate, 11 mM magnesium acetate, 10 mM dithiothreitol (buffer C); 75 mM phosphoenolpyruvate; 1 mM ATP, 0.3 mM GTP, CTP, and UTP; 0.1 mM folinic acid; 0.13 mg of stripped tRNA; 0.1 mM of each of the 20 common amino acids; approximately 200 μg of supernatant; 30 μg of crude initiation factors; 20 μg of T4-DNA; 0.25 mg of ribosomes; and 7.5 μCi of [^{3}H]leucine in 0.2 ml (Gold and Schweiger, 1969). Mixtures were incubated at 37° for 25 min.

TABLE IV: Some Properties of Phosphorylatable 30S Proteins.

Protein	Stoichiometry[a]	Residue Phosphorylated	Topography	Functional Properties
9 (S4)	Unit	Thr (+Ser)	Reacts with SH reagents[b]	Binds to 16S RNA.[e] Ram protein.[f] Required for assembly. Stimulates all functions.[g]
5 (S9)	Unit	Ser	Trypsin sensitive.[c] Reacts with bisimidoesters.[d]	Split protein.[h] Required for assembly. Stimulates all functions.[g]
2b (S18)	Fraction		Reactive with SH reagents[b] and methoxy-5 nitropone.[c]	Required for optimal activity. Stimulates all functions.[g]
2a (S19)	Unit	Thr (+Ser)	Trypsin sensitive.[c] Reacts with bisimidoesters.[d]	Required for optimal activity. Stimulates all functions.[g]

[a] Traut *et al.* (1969); Kurland *et al.* (1969). [b] Craven and Gupta (1970); Moore (1971). [c] Chang and Flaks (1970). [d] Bickle *et al.* (1972). [e] Mizushima and Nomura (1970); Shaup *et al.* (1970). [f] Zimmerman *et al.* (1971). [g] Nomura *et al.* (1969). [h] Traub *et al.* (1967).

and in the demonstration of the same pattern of specificity toward phosphorylation of the components isolated from intact ribosomes and for pure 30S ribosomal proteins strongly suggests the receptors in all cases to be the ribosomal proteins indicated.

Consistent with the existence of a linkage to protein and not RNA was the fact that the incorporated phosphate was released by mild alkaline hydrolysis but was stable to mild acid. Finally, serine phosphate and threonine phosphate were identified as the phosphorylated residues.

The identification of the phosphorylated 30S proteins 9 (S4), 5 (S9), 2a (S19), and 2b (S18) was made by gel analysis and radioautography and confirmed in three cases with the individual pure proteins. Identification of 2b (S18) and the 50S proteins was made only from electrophoretic behavior in two systems. The identity of the major 50S protein phosphorylated in the intact subunit is unambiguous: protein 13–14 X (L2) is the only protein of molecular weight 36,000.

Some properties of the four major 30S phosphate-accepting proteins are given in Table IV. Three of the proteins are present in amounts equimolar with the ribosome particle and one is a fractional protein. Two of the proteins are required for assembly and one binds directly to the 16S RNA. All of the proteins are attacked by certain chemical reagents or by trypsin although protein 9 (S4) is relatively protected. Patently, the proteins are accessible to the catalytic subunit of protein kinase, an enzyme of mol wt 43,000 (Krebs, 1972). The fact that the same four proteins are phosphorylated after the ribosome structure is destroyed with ribonuclease indicates that the steric position of the proteins in the particle is not a primary factor in determining the specificity of phosphorylation. This must therefore be a property of the primary structure of the proteins and the specificity of the protein kinase. On the other hand, the extent of phosphorylation of proteins 2a (S19) and 2b (S18) increased when the ribosome structure was disrupted.

Protein 5 (S9) is the major substrate for protein kinase in the 30S subunit and accepts 70–80% of the total phosphate incorporated. This corresponds to 0.3–0.4 mole of phosphate per particle or per mole of protein 5 (S9) on the assumption of one copy of 5/particle. Even with the free proteins

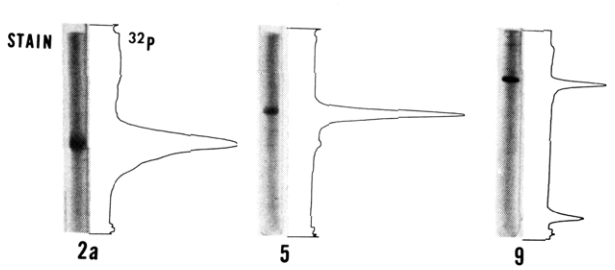

FIGURE 5: Identification of phosphorylated 30S ribosomal proteins. Samples containing 5 μg of 16 pure 30S ribosomal proteins were phosphorylated, electrophoresed in 7.5% urea gels (pH 4.5) and stained with Coomassie Brilliant Blue. The stained patterns of the three proteins incorporating radioactive phosphate, proteins 2a, 5, and 9, are on the left in each figure; the spectrophotometric scans of the radioautograms are on the right.

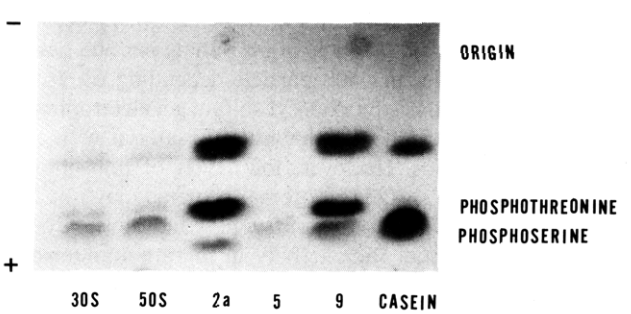

FIGURE 6: Identification of phosphoserine and phosphothreonine in phosphorylated ribosomal proteins. Total 50S and 30S ribosomal proteins, individual 30S proteins, and casein were phosphorylated, and subjected to partial acid hydrolysis, and thin-layer electrophoresis was performed at pH 1.9 as described in Methods. The intensity of the radioactive band in protein 5 moving as phosphoserine was reduced since the sample contained 10% of the material present in the other purified samples. Radiaoctive residues were visualized by radioautography, and the position of authentic phosphoserine and phosphothreonine added as carrier for each sample was determined by staining with ninhydrin.

or with single pure proteins equimolar amounts of phosphate were not incorporated. Since attempts to increase the level of phosphorylation by altering reaction conditions and increasing the time of incubation were unsuccessful, alternative explanations for incomplete phosphorylation must be sought. These include the possibility that the proteins may not be homogeneous in primary structure or conformation, thus blocking phosphate receptor activity; alternatively, phosphate already present on a fraction of the protein would decrease the total acceptor activity *in vitro*.

The effect on ribosomal activity by phosphorylation was measured with a T4-DNA-linked protein-synthesizing system (Gold and Schweiger, 1969). Under conditions of 50% phosphorylation (0.5 mole of phosphate/70S particle) the amino acid incorporation was reduced 25% compared to the nonphosphorylated control. Sucrose density gradient centrifugation of the reaction mixtures which contained phosphorylated ribosomes showed that phosphorylated proteins were present in both the 70S and polysome regions; however, fewer polysomes were present compared to the nonphosphorylated control, consistent with the decrease in activity measured by amino acid incorporation. Since a large number of proteins are involved in phosphorylation, it is not possible at this time to assign an inhibitory function to any specific protein.

In addition to phosphorylated ribosomal proteins, proteins loosely associated with the ribosome and removed from it with 0.5 M ammonium chloride have substantial activity as substrates for the protein kinase. One of these proteins has a molecular weight consistent with its identity as initiation factor F-2 and recent experiments with the pure factor have confirmed that F-2 is phosphorylated (J. Fakunding, J. A. Traugh, R. R. Traut, and J. W. B. Hershey, manuscript in preparation).

The observation that bacterial ribosomal proteins and proteins in the high salt wash fraction are phosphorylated by a mammalian protein kinase in a highly specific manner raises the question of whether these proteins are also phosphorylated *in vivo* by a protein kinase from *E. coli*. There is no unambiguous answer to the question at this time. A cAMP-dependent protein kinase from *E. coli* has been reported (Kuo and Greengard, 1969). Phosphoserine has been reported in a number of procaryotes although the amounts are significantly lower than in eucaryotes (Forsberg, *et al.*, 1969). Gordon (1971) examined the phosphorylation of ribosomal proteins and elongation factors in growing *E. coli* cells. He found between 0.1 and 0.7 mole of phosphate per 30S particle and 0.8 to 3.7 moles per 50S particle depending on growth conditions, but analysis by dodecyl sulfate gel electrophoresis did not show coincidence of the labeled material with ribosomal protein bands. However, low levels of phosphorylation of one or two specific ribosomal proteins may not have been detected under his experimental conditions. Gordon also found substantial radioactivity in the ammonium chloride high salt wash fraction which is consistent with our results.

If phosphorylation of either ribosomal proteins or proteins loosely associated with the ribosome, such as initiation factors, is important in the regulation of protein synthesis, low levels of phosphorylation could be significant. First, only a few specific protein species might be involved, and second, these proteins might undergo a phosphorylation–dephosphorylation cycle during their functioning in translational control. The specific phosphorylation of bacterial ribosomal proteins with the mammalian kinase reported here and the possible involvement of phosphorylated ribosomal proteins in a translational regulatory mechanism make it important to investigate the possible formation of phosphorylated ribosomal proteins in bacteria *in vivo*.

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Use of Nonspecific Dye Labeling for Singlet Energy-Transfer Measurements in Complex Systems. A Simple Model†

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ABSTRACT: Techniques are described which will permit the semiquantitative interpretation of singlet–singlet energy-transfer measurements on multiply labeled single proteins and protein complexes. The two critical assumptions are that fluorescent chromophores can be placed anywhere at random on a protein surface and that the stoichiometry of labeling is governed by a Poisson distribution. In calculations on protein complexes it is further assumed that one can exclusively localize donors on the surface of one protein, and acceptors on another. Calculations were initially carried out to allow for the occurrence of donor–donor energy transfer. These show that in most cases of interest one can neglect this process. Singlet energy-transfer measurements on a double-labeled protein will permit measurement of the anhydrous radius. The results are quite insensitive to variations in axial ratio if the volume is kept constant. In a complex of two or more proteins, singlet energy-transfer measurements will enable fairly accurate determination of the distances between pairs of proteins. For most accurate results in both cases one should attempt to work at ratios of several acceptor molecules per protein. This minimizes errors due to uncertainties in the extinction of bound dyes. The average number of donor molecules per protein is not important. Examples of the application of these calculations are given in the accompanying paper.

Fluorescence techniques have yielded much useful information about the structure of biopolymers (Cantor and Tao, 1971; Stryer, 1968). Both intrinsic protein fluorescence (from aromatic amino acids) and extrinsic fluorescence (from dye–protein conjugates or prosthetic groups) have been valuable. A number of enzymes bind dyes specifically at the active site (Glazer, 1970). This fact has been fully exploited in the numerous experiments using fluorescent dyes to probe the nature of the active site (or binding site), its polarity, and the disposition of aromatic amino acids nearby (see for example Stryer, 1965; DeLuca, 1969; Chen and Kernohan, 1967; Brand *et al.*, 1967; McClure and Edelman, 1967; Turner and Brand, 1968). Energy-transfer experiments have generally, though not exclusively, been restricted to these situations where the transfer from tryptophan to a fluorescent probe at a specific site is observed.

It is our purpose to demonstrate the feasibility of obtaining useful information on proteins randomly labeled with fluorescent donor and acceptors. Somewhat analogous calculations have been reported in the past by Teale and his coworkers (Badley and Teale, 1969, 1971; Dale and Teale, 1970). However, they were aimed at different kinds of chromophore distributions. We shall make three assumptions. The stoichiometry of dye labeling is governed by the Poisson distribution. The spatial location of dyes is anywhere at random on the protein surface. If more than one protein is present one can control which protein has which dye by separate labeling and subsequent reconstitution. The success of this approach is demonstrated by the experimental results given in the accompanying paper (Gennis *et al.*, 1972). The procedures we describe should find general use in studies on such diverse and complicated systems as ribosomes, membranes, multiprotein complexes, and the cell surface.

Theory

Förster transfer has been shown to apply to the systems we will be discussing (Latt *et al.*, 1965; Stryer and Haugland, 1967). The energy from an excited donor is transferred *via* a dipole–dipole nonradiative mechanism to an acceptor nearby (Förster, 1965). The distance over which this occurs may be as much as 60 Å or more, making it a valuable tool for the study of macromolecules. Förster's equation relates the absolute rate of transfer, k_t, to the spectral characteristics of the donor and acceptor, their relative orientation, and the distance between them.

$$k_t = \frac{1}{\tau} \times \text{constant} \times \frac{\kappa^2 \phi_f J}{n^4 r^6} \text{ sec}^{-1} \quad (1)$$

$$k_t = \frac{1}{\tau}(R_0/r)^6 \quad (2)$$

where R_0 = (constant $\times$ $\kappa^2 \phi_f J/n^4)^{1/6}$, κ^2 is the dipole–dipole orientation factor, ϕ_f is the fluorescence quantum yield of the donor in the absence of transfer, n is the refractive index of

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