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Extracellular Labeling of Growing Secreted Polypeptide Chains in *Bacillus subtilis* with Diazoiodosulfanilic Acid†

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ABSTRACT: Studies of the mechanism of protein secretion in a Gram-positive bacterium, *Bacillus subtilis*, yielded results very similar to those previously obtained with a Gram-negative organism: nascent chains protruding from protoplasts could be labeled extracellularly; the labeled chains could be recovered on polysomes isolated from the membrane–polysome fraction; they could be released by puromycin, low Mg^{2+}, or chain completion; the completed chains include a known secreted protein (α-amylase); and the ribosomes appear to be attached to membrane solely by their nascent chains. The reagent used for extracellular labeling, [^{125}I]diazoiodosulfanilic acid, yielded severalfold more specific labeling of the nascent chains (7–10% of the total cellular labeling and one-fourth to one-third of that of the membrane–polysome fraction) than was obtained earlier with another nonpenetrating reagent.

The presence of ribosomes bound to the endoplasmic reticulum, in animal cells that secrete proteins, suggested that these proteins cross the membrane as growing chains (reviewed by Palade, 1975). This model was supported by the finding that extracts of a myeloma cell synthesized an immunoglobulin chain as a larger precursor, with an additional N-terminal "signal" sequence (Milstein et al., 1972) of predominantly hydrophobic residues (Schechter et al., 1975). The formation of such a precursor, its cleavage by an enzyme in the membrane, and in many cases the sequence of the additional segment have now been demonstrated for many proteins secreted by animal cells (e.g., Blobel & Dobberstein, 1975; Devillers-Thiery et al., 1975) and more recently for proteins secreted by bacteria (Inouye et al., 1977; Inouye & Beckwith, 1977). These findings strongly suggested that such signal segments (Blobel & Sabatini, 1971) play a role in initiating secretion, but they do not establish whether the chain that follows is folded and released from the ribosome before or after transfer across the membrane. With spheroplasts of *Escherichia coli* we were able to demonstrate directly that a periplasmic protein is secreted as a growing chain (Smith et al., 1977): such chains could be extracellularly labeled with a reagent ([^{35}S]acetylmethionyl methyl phosphate sulfone, AMMP[1]) that acylates amino groups. Moreover, in this organism polysomes appear to be attached to membrane solely via nascent chains (Smith et al., 1978a), in contrast to results reported for animal cells (Adelman et al., 1973; Sabatini & Kreibich, 1976) and for chloroplasts (Chua et al., 1976).

The present work extends these studies, with very similar results, to a Gram-positive organism, *Bacillus subtilis*. In addition, since only 2% of the bound label was attached to growing chains in our studies with AMMP (Smith et al., 1977), we have explored the use of a reagent, diazotized [^{125}I]iodosulfanilic acid (DSA), that reacts only with histidine and tyrosine residues (reviewed by Carraway, 1975) and have obtained three- to fourfold increase in the specificity of labeling.

Materials and Methods

Bacteria and Protoplasts. *B. subtilis* cells of ATCC strain 6051a, constitutive in the synthesis of α-amylase, were grown at 37 °C with vigorous aeration (unless otherwise indicated) in minimal medium A (Davis & Mingioli, 1950) supplemented with 0.4% glucose and 0.2% casamino acids. To stabilize polysomes chloramphenicol (200 μg/mL) was added to 25 mL of an exponentially growing culture (5×10^8 cells/mL), which was poured onto excess ice. The cells were pelleted by brief centrifugation in the cold and resuspended in 2 mL of 100 mM Tris-HCl, pH 8.0, with 25% w/v sucrose. Protoplasts were formed by adding 400 μg of lysozyme and incubating at 37 °C for 10 min. Conversion was usually greater than 95% as monitored by microscopy.

Extracellular Labeling of Protoplasts. The protoplasts were centrifuged and resuspended in 0.5 mL of 10 mM NaH_2PO_4, pH 7.5, with 25% sucrose. Unless otherwise indicated, 50 μCi of [^{125}I]DSA (specific activity ~2000 Ci/mmol) was dried under vacuum, converted to the diazonium salt by incubation for 10 min at 0 °C with 10 μL of 0.05 mM $NaNO_2$ and 10 μL of 1 mM HCl, and added to the protoplast suspension derived from 1.2×10^{10} cells (final DSA concentration, 6 μM). After incubation for 15 min at 0 °C with gentle shaking, the

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[1] Abbreviations used: AMMP, acetylmethionyl methyl phosphate sulfone; DSA, diazoiodosulfanilic acid; $NaDodSO_4$ gel electrophoresis, gel electrophoresis containing 10% polyacrylamide, 0.1% sodium dodecyl sulfate in 0.1 M phosphate buffer; EF-G, bacterial elongation factor G; RRF, ribosome release factor; buffer A, 10 mM Tris-HCl, pH 7.6, 50 mM KCl, 10 mM $Mg(OAc)_2$.

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Table I: Distribution of Label in *B. subtilis*[a]

fraction	[^{125}I]DSA (cpm × 10^{-5})	[^{3}H]uracil (cpm × 10^{-4})	[^{14}C]oleate (cpm × 10^{-3})
protoplasts	30	24	200
membrane-polysome complexes	8	5	70
derived polysomes, freed of membrane by DOC	2	4.4	0.50
free polysomes	0.22	16	0.75

[a] As described in Materials and Methods, cells were labeled with [^{14}C] oleic acid and [^{3}H] uracil for two generations, blocked in protein synthesis by chloramphenicol and chilling, and converted to protoplasts. In a parallel culture unlabeled protoplasts were labeled with [^{125}I] DSA at 6 μM. Unlysed protoplasts and aggregates were removed by centrifugation, the supernatants were fractionated, and the radioactivity was assayed. Very similar results on labeling with DSA were obtained in several repeated experiments.

protoplasts were pelleted, washed three times by resuspension in 25% sucrose in buffer A (10 mM Tris-HCl, pH 7.6, 50 mM KCl, 10 mM $Mg(OAc)_2$), and lysed by suspension in 2 mL of buffer A. Electrophoretically pure DNase (5 μg/mL) was added, and unlysed protoplasts and debris were removed by centrifugation at 3000*g* for 10 min.

Preparation of Membrane-Associated, Derived, and Free Polysomes. Membrane-associated and free polysomes were prepared by passage through a Sepharose 2B column (Smith et al., 1978a) since the discontinuous density gradient method used with *E. coli* lysates (Smith et al., 1977) did not adequately separate the two kinds of polysomes from *B. subtilis*. Derived polysomes (i.e., freed of membrane) were prepared by washing the membrane–polysome fraction three times with a 1% sodium deoxycholate solution in buffer A (Smith et al., 1977).

Reagents. Commercial α-amylase, which migrated as a single band on $NaDodSO_4$–polyacrylamide gels, was obtained from Worthington Biochemicals. Rabbit antiserum to it was prepared by standard methods (see Furano, 1975) and yielded a single precipitin line by immunodiffusion. [^{125}I]Iodosulfanilic acid, [^{14}C]oleic acid, ^{14}C-labeled amino acids, and [^{3}H]uracil were obtained from New England Nuclear Corp. All other chemicals were of reagent grade. [^{125}I]Iodosulfanilic acid of high specific activity was used since membrane integrity is impaired by the extensive derivatization that would be required with a preparation of low activity (Carraway, 1975).

Results

Cell Fractionation. Cells grown for two generations with [^{14}C]uracil and [^{3}H]oleic acid, to label ribosomes and membrane lipid, respectively, were converted to protoplasts and lysed, and the membrane–polysome complexes and the free polysomes were separated, as described in Materials and Methods. As Table I shows, the middle fraction, labeled "membrane–polysome complexes", contained ca. 30% of the total RNA and 35% of the total lipid. This fraction was continuous with a preceding, discarded fraction, at the void volume, which evidently consisted largely of free membrane, since it could be calculated (by difference from the other fractions; Table I) to contain ca. 65% of the cellular lipid but less than 10% of the RNA. The free polysomes and ribosomes eluted later from the column: this fraction contained ca. 65% of the cellular RNA and was contaminated with about 1% of the lipid.

Extracellular Labeling of Nascent Chains. The protoplasts were treated with [^{125}I]DSA (50 μCi/1.2 × 10^{10} cells) at the concentration recommended by the vendor for extracellular labeling of erythrocytes (6 μM). Under the conditions used, 10–15% of the radioactivity was attached to the protoplasts. As Table I shows, the membrane–polysome fraction contained ca. 33% of the total ^{125}I in the protoplasts. After three washings with deoxycholate, which removes more than 99% of the membrane (as shown in parallel experiments with [^{3}H]oleic acid; Table I), the derived polysomes, now eluting as free polysomes from the Sepharose column, retained ca. 7% of the total extracellular label. This result represents a threefold enrichment over the extracellular labeling obtained with [^{35}S]AMMP in *E. coli* spheroplasts (Smith et al., 1977). Similarly, when *E. coli* spheroplasts were labeled with [^{125}I]DSA at this concentration, 8–10% of the total cellular label was attached to the derived polysomes (unpublished).

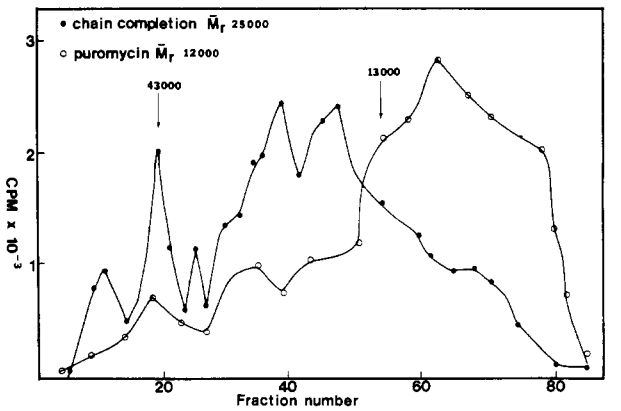

FIGURE 1: Increase of M_r of ^{125}I-labeled peptides on chain completion. To release nascent peptides 1 A_{260} unit of labeled membrane-derived polysomes (ca. 40 000 cpm) was incubated in 0.1 mL of buffer A with puromycin (200 μg/mL), EF-G (100 μg/mL), and GTP (500 μg/mL) for 30 min at 37 °C. A similar sample was incubated in a protein-synthesizing system to allow chain completion and release, as described (Smith et al., 1977). Both the released nascent peptides (○) and the completed peptides (●) were analyzed by disc gel electrophoresis with 0.1% $NaDodSO_4$, as before (Smith et al., 1977).

In *E. coli* the free polysome fraction was devoid of label from DSA (unpublished), as previously observed with AMMP (Smith et al., 1977). With *B. subtilis*, in contrast, that fraction contained about 3% as much label from DSA as did the membrane–polysome complexes (Table I). However, at this DSA concentration the label in the free polysome fraction appears to be largely due to contamination with membrane-bound polysomes, rather than to penetration of DSA, for the same fraction also contained ca. 1% as much [^{14}C]-oleate as the complexes (Table I), and a single deoxycholate treatment solubilized more than 75% of both labels (data not shown). It thus appears that in *B. subtilis* lysates, unlike those of *E. coli*, some polysomes are associated with only small fragments of membrane and, hence, are recovered in the free polysome fraction.

Evidence for Attachment of Extracellular Label to Polysomes as Nascent Chains. With the extracellularly labeled polysomes, after removal of membrane by deoxycholate, the location of the label on nascent chains was demonstrated by the same tests previously used with *E. coli* (Smith et al., 1977): i.e., 75% more of the ^{125}I was released by treatment with puromycin (together with GTP and EF-G), or by dialysis against 10^{-5} M Mg^{2+}, or by completion of the nascent peptide chain. Moreover, completion of the ^{125}I-labeled chains in a protein-synthesizing system markedly increased their mean M_r, compared with the chains released by puromycin. Because of the close similarity to the results obtained with *E. coli* and AMMP (Smith et al., 1977), only the last point is documented (Figure 1).

Table II: Labeling of *B. subtilis* Protoplasts with [^{125}I]DSA[a]

fraction	labeling (cpm × 10^{-5}) at various concn of [^{125}I]DSA			
	6 μM	30 μM	60 μM	200 μM
protoplasts	29	83	122	328
membrane-polysome complexes	10	27	56	139
derived polysomes (deoxycholate washed 3×)	3.2	8.2	29	70
free polysomes	0.8	4.5	14	40
deoxycholate-washed (1×) free polysomes	0.15	1.8	9.2	29
washed free polysomes treated with puromycin	0.01	0.5	4.6	22.2

[a] Protoplasts were labeled with [^{125}I]DSA at the concentration indicated and were fractionated and assayed for radioactivity as described in Materials and Methods. Incubation with puromycin was as in Figure 1.

Variable Concentration of Labeling Reagent. Because the number of chains labeled per cell was small (see Discussion), and because the protoplasts provide ca. 10 times as much surface per unit weight as erythrocytes, we explored the possibility that higher concentrations of the reagent might be tolerated without membrane damage. However, the amount of penetration was found to increase with increasing DSA concentration. As Table II shows, with an increase in concentration from 6 to 30 μM, there was indeed increased labeling of the membrane-derived polysomes, but there was a much greater increase in labeling of the free polysome fraction, to a level roughly half that of the derived polysomes. Moreover, with increasing DSA concentrations, from 6 to 200 μM, deoxycholate removed less and less of the label from the free polysome fraction, and subsequent puromycin treatment also removed less and less of the residual label. This label, after the two treatments, presumably resides largely on free polysomes reached by penetrating reagent: the value was negligible at 6 μM DSA but rose from 6% to 30% of the level of label of the derived polysomes at 30 to 200 μM DSA, respectively. (It should be noted that the free polysomes are three times as numerous as the membrane-associated polysomes.) Moreover, at 200 μM DSA the ribosomes in the membrane–polysome fraction were heavily labeled, for puromycin released only 20% of the label on the derived polysomes (data not shown), compared with 75% after labeling at 6 μM DSA. We conclude that higher concentrations of DSA can be used to achieve higher levels of extracellular labeling of the nascent chains on membrane-associated polysomes, but the contamination by labeling of the ribosomes is substantial.

Immunological Detection of Labeled Nascent Chains of α-Amylase. Figure 1 shows that the chains completed by the derived polysomes in vitro included a discrete peak at M_r ca. 43 000. This result suggested the formation of α-amylase, a major secreted protein of the strain being used. Direct evidence for the extracellular labeling of this growing protein was therefore sought.

The extracellularly ^{125}I-labeled polysomes, freed of membrane, were allowed to release their chains by completion or by treatment with puromycin, and the products were reacted in agar diffusion with antiserum to α-amylase. As Figure 2 shows, the labeled products of chain completion formed a precipitin line with this antiserum. A slight line is also seen for the puromycin-released labeled material, suggesting that some incomplete chains of α-amylase react with the antiserum.

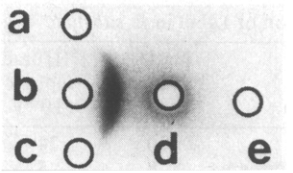

FIGURE 2: Immunological detection of α-amylase. After chain completion and release of the ^{125}I-labeled peptides, the ribosomes were removed by centrifugation. Amylase was identified by radioimmunodiffusion performed with microslides containing 1 mL of 1.5% agar in buffer (0.14 M NaCl, 0.1 M Na_2HPO_4, pH 7.4). The wells contained (a) puromycin-released ^{125}I-labeled chains, (b) antiserum to α-amylase, (e) normal serum, (d) completed ^{125}I-labeled peptides, and (c) *E. coli* periplasmic proteins labeled in solution with [^{125}I]DSA. Wells a, c, and d each contained ca. 10 000 cpm. Precipitin lines were allowed to develop for 4 h at 37 °C in a humid chamber. The slides were washed extensively with the above buffer and the gel was transferred to filter paper and dried under vacuum. The dried gel was then exposed to Kodak X-ray film for 1 day.

Table III: Release of ^{3}H-Labeled Ribosomes from the Membrane-Polysome Fraction of *B. subtilis*[a]

treatment	% released
0 °C, 30 min	10 ± 8
37 °C, 30 min	15 ± 11
puromycin, 37 °C, 30 min	73 ± 21
RNase, 0 °C, 30 min	28 ± 14

[a] Membrane-polysome complexes with ^{3}H-labeled ribosomes (ca. 5000 cpm) were incubated for 30 min at the temperature noted, in buffer A, or with puromycin plus EF-G and GTP as in Figure 1, or with RNase (30 μg/mL). The assay for release of ribosomes from membrane has been described (Smith et al., 1978a). Data are the mean (and the range) of five to ten separate experiments.

The controls were negative. To confirm the identification of the enzyme, completed labeled chains were precipitated by antiserum from solution and were analyzed for M_r in the presence of $NaDodSO_4$, as previously described (Smith et al., 1977). The expected peak at 43 000 was observed, plus smaller peaks that presumably reflected proteolysis and incomplete chains (data not shown).

Effect of Puromycin or RNase on Attachment of Ribosomes to Membrane. In *E. coli* lysates, ribosomes appear to be attached to membrane solely by their growing chains, since puromycin treatment, under a wide range of ionic conditions, released over 75% of the ribosomes, and much of the residue could be accounted for as ribosomes that failed to react with puromycin (Smith et al., 1978a). Because this result differs from those reported for eukaryotic cells, and because the cell envelopes differ substantially in Gram-positive and in Gram-negative bacteria, it seemed advisable to study the mode of attachment in *B. subtilis*. The results, obtained with membrane–polysome complexes containing uracil-labeled ribosomes, were similar to those observed with *E. coli*: treatment with puromycin (plus EF-G and GTP) released ca. 75% of the ribosomes from membrane (Table III). Moreover, as with *E. coli*, the retention of some ribosomes by membrane after puromycin treatment could be largely explained by the failure of puromycin to release their chains: when cells were pulse-labeled in the nascent chains, and their membrane-bound polysomes were treated with puromycin and then washed with deoxycholate to remove freed chains attached to membrane, the residual label per ribosome was about 75% of that observed in the untreated complexes. It should also be noted that the incomplete reaction with puromycin is not special for membrane-bound polysomes: the same range has been observed in many experiments with cytoplasmic ribosomes (data not shown).

As expected, the polysomes freed by puromycin remained relatively intact (data not shown), since the purified complexes lack the factor (RRF) required for release of ribosomes from mRNA after loss of peptide (Hirashima & Kaji, 1972; Kung et al., 1977). Accordingly, mRNA does not appear to be responsible for ribosome attachment, except indirectly through the formation of nascent chains.

Table III also demonstrates the presence, on membrane-bound polysomes, of some ribosomes that appear to be attached to membrane only indirectly, via mRNA, since they could be released by cleaving the mRNA of polysomes by mild RNase treatment. The release, corrected for the 10% loss in controls incubated without enzyme, suggests that such "danglers" were about 20% of the bound ribosomes. These are presumably ribosomes that have not yet inserted their nascent chains into membrane or ribosomes forming a cytoplasmic protein but linked polycistronically with mRNA for a protein entering the membrane.

Discussion

In this paper *B. subtilis* cells were fixed in protein synthesis by chloramphenicol, converted to protoplasts, treated with an ^{125}I-labeled reagent (DSA) that attaches to extracellular tyrosine and histidine residues, and fractionated. The polysomes of the membrane–polysome fraction, freed of membrane components by exhaustive washing with deoxycholate, contained about 25% of the label of that fraction, corresponding to 7% of the total label on the protoplasts (Table I). With *E. coli* spheroplasts an even higher proportion of the total cellular label (10%) was shown by several tests, as in *E. coli* (Smith et al., 1977), to be attached to nascent chains. Moreover, when these labeled chains were completed one of the products could be identified, serologically and by M_r, as a major secreted protein, α-amylase.

In our earlier studies, with a reagent ([^{35}S]AMMP) that labels amino groups of phosphatides as well as of proteins, only ca. 2% of the cellular label in *E. coli* was found on polysomes (Smith et al., 1977). It is clear that with DSA the specific labeling of nascent secreted chains, compared with the background labeling of membrane components, is substantially higher. At higher concentrations this reagent penetrated the membrane and caused significant labeling of ribosomes, as previously observed with animal cells (Carraway, 1975), but at 6 μM, used in the experiments summarized above, penetration was not significant. At this concentration there was slight labeling of the free polysome fraction in *B. subtilis* (but not in *E. coli*). However, this level (ca. $^1/_{40}$ that in the membrane-derived polysome fraction; Table I) is too low to interfere with identification of extracellularly labeled nascent chains. Moreover, even that slight labeling appears to be mainly due not to penetration but to incomplete separation of the membrane-bound and the free polysomes from *B. subtilis*, since the latter fraction was contaminated with membrane labeled with [^{14}C]oleic acid. It thus appears that in the lysis of *E. coli* spheroplasts all the bound polysomes were attached to large fragments of membrane, whereas lysis of *B. subtilis* protoplasts under identical conditions yielded some bound polysomes with only small amounts of membrane attached.

Though the nascent chains contained one-fourth to one-third of the label in the membrane–polysome fraction of *B. subtilis* (Table I), these values corresponded, in various experiments, to only 20–80 molecules of DSA per cell or about twice the value observed earlier with AMMP in *E. coli* spheroplasts (Smith et al., 1977). Since the ribosomes recovered in the membrane–polysome fraction were one-fourth of the total (Table I), or several thousand per cell, it seems clear that under the conditions used only a small fraction of the growing chains are being labeled. Efforts to saturate the labeling of the chains, at higher DSA concentrations, were not promising, because of increasing penetration of the reagent: with the DSA concentration increased 35-fold the labeling of the membrane-derived polysomes increased 20-fold, but the total deoxycholate-washed polysomes from the free polysome fraction now showed nearly half as great labeling as those from the membrane (Table II). Moreover, at the highest DSA concentration the free polysomes, after washing with deoxycholate and treatment with puromycin, retained ca. 80% of the ^{125}I label, suggesting that under these conditions most of the label is attached directly to the ribosome.

We suggested earlier (Smith et al., 1977) that the low level of labeling of nascent chains of *E. coli* spheroplasts with AMMP might be due to blocking by outer membrane. However, with *B. subtilis* the ratio of nascent chains to surface proteins labeled and DSA is quite high (ca. 25%) and does not imply any problem of access. Accordingly, the low level of total labeling of the chains simply reflects the kinetics of reaction of the tolerable concentration of the reagent with all groups on the protoplast surface.

Our demonstration of the secretion of nascent chains in both *E. coli* and *B. subtilis* supports the view that this mode of secretion is quite general. Moreover, in animal cells a viral membrane protein also is clearly inserted as a growing chain, since glycosylation by a membrane enzyme occurs only during chain elongation (Katz et al., 1977; Rothman & Lodish, 1977; Toneguzzo & Ghosh, 1978).

The release of ribosomes from membrane by puromycin indicates that, in *B. subtilis*, as in *E. coli* (Smith et al., 1978a), the ribosomes in osmotic lysates must virtually all be attached either to open membrane fragments or to "inside-out" vesicles, rather than being sealed within "right-side-out" vesicles. Randall & Hardy (1977) have reported that disruption of *E. coli* cells by sonication yields complexes composed largely of open membrane fragments with few sealed vesicles.

With the preparations from *B. subtilis* (Table III), as with those of *E. coli* (Smith et al., 1978a), puromycin released the ribosomes from membrane (as intact polysomes), except for those ribosomes that failed to react with puromycin, and release by RNase suggested that a small proportion of the ribosomes is attached to membrane only indirectly, via an mRNA shared with a ribosome carrying a chain that is entering the membrane. The puromycin effect extends to a Gram-positive organism the evidence (Smith et al., 1978a) that in bacteria ribosomes are not attached to membrane directly, as reported for animal cells (Adelman et al., 1973; Sabatini & Kreibich, 1976) and for chloroplasts (Chua et al., 1976), but are attached solely via the nascent chain (see also Aronson, 1966). The apposition is evidently very close since Pronase treatment of the membrane–polysome complexes of *B. subtilis*, under conditions optimizing proteolysis of nascent chains, did not release the ribosomes from the membrane (Smith et al., 1978b,c). Accordingly, the length of the nascent chain between the ribosome and the membrane must be very short or protected.

The evidence against a direct attachment of ribosome to membrane speaks against a possible secretory mechanism in which the energy of chain elongation on the ribosome would push the growing chain through the membrane. An alternative possibility therefore becomes more attractive: that the secreted chain, despite its hydrophobic leader segment, does not simply slide through the lipid of the membrane but passes through

a specific channel, within a machinery that transduces metabolic energy into unidirectional transfer of the growing chain.

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Cation-Induced Regulatory Mechanism of GTPase Activity Dependent on Polypeptide Initiation Factor 2†

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ABSTRACT: Initiation factor IF-2 ribosome dependent GTP hydrolysis (uncoupled GTPase) presents a bell-shaped pH profile which is shifted by changes in ionic strength. At low ionic strength (I = 25 mM) the maximal hydrolytic activity occurs at pH 7.5; when the ionic strength is increased the pH optimum of the reaction is shifted toward more acidic values. Such behavior can be satisfactorily explained as the effect of an electrostatic potential developed by a neighboring polyanion, presumably RNA, on the catalytic site. The addition of fMet-tRNA^{fMet} or AcPhe-tRNA^{Phe} and messenger RNA (coupled GTPase) changes the ionic strength–pH characteristics of the reaction. Thus there is an effect, direct or indirect, of components located at the ribosomal P site. Investigation of the effect of neighboring polyanions on the catalytic activity of the factor-dependent ribosomal GTPases can be seen to provide information about their functional significance that is complementary to that gained from direct structural studies.

Ribosomes are essential elements of GTPase activities developed by initiation factor IF-2 as well as elongation factors EF-G and EF-Tu during *Escherichia coli* protein synthesis in vitro (Kolakofsky et al., 1969; Lelong et al., 1970; Dubnoff & Maitra, 1972; Stöffler & Wittmann, 1977; Grunberg-Manago et al., 1978).

GTP appears to interact directly with these factors, at least with the last two (Miller & Weissbach, 1977; Brot, 1977; Grunberg-Manago et al., 1978). However, at present, it is not clear whether the catalytic site for GTP hydrolysis is located on the ribosome or on the protein factor itself. For EF-Tu, it has been shown that the elongation factor is capable of developing GTP hydrolytic activity in the presence of the antibiotic kirromycin (Chinali et al., 1977). Isolated ribosomal protein L7/L12 also seems to be able to induce an elongation factor Tu dependent GTPase activity (Donner et al., 1978).

It has also been shown that EF-G and EF-Tu dependent GTPase activities were very sensitive to monovalent cation variations such as K^+ or NH_4^+ (Voigt et al., 1974; Arai &

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