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Protein Synthesis Kinetics with Ribosomes from Temperature-Sensitive Mutants of *Escherichia coli*†

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ABSTRACT: The kinetics of MS2 ribonucleic acid (RNA) directed protein synthesis have been investigated at seven temperatures between 30 and 47 °C by using ribosomes isolated from a wild type strain and seven temperature-sensitive mutants of *Escherichia coli*. The amount of MS2 coat protein formed at each temperature was determined by gel electrophoresis of the products formed with control ribosomes. With ribosomes from each of the mutant strains, the activation energy required to drive protein synthesis below the maximum temperature (up to 40 °C) was increased relative to the control (wild type) activity. Preincubation of the ribosomes at 44 °C revealed the kinetics of thermal inactivation, with ribosomes from each of the mutants having a half-life for inactivation less than that of the control ribosomes. A good correlation was observed between the relative activity of the different ribosomes at 44 °C and their relative rate of thermal inactivation. Mixing assays allowed the identification of a temperature-sensitive ribosomal subunit for each of the mutants. Defects in one or more of three specific steps in protein synthesis (messenger RNA binding, transfer RNA binding, and subunit reassociation) were identified for the ribosomes from each mutant. The relationship between temperature sensitivity and protein synthesis in these strains is discussed.

The biosynthesis of protein in cells involves a complex series of reactions beginning with free amino acids and ending with a structurally complete and functionally active macromolecule. The intermediate steps between these two states have been systematically investigated by a large number of workers [see the review by Weissbach & Pestka (1977)]. Although protein synthesis is a very complex activity, involving numerous cooperative elements, in vitro it can be analyzed kinetically and thermodynamically as a simple two-state process, going from free amino acids to polypeptide (Spirin, 1978). A critical component for this transition is the ribosome. Its structural and functional contributions to the process of protein synthesis have been extensively examined by a variety of biochemical techniques and assays (Nomura et al., 1974). A recent approach to the study of the involvement of the multiple macromolecular components of the ribosome in protein formation has been the isolation of a collection of temperature-sensitive mutants of *Escherichia coli*, defective in protein synthesis at 44 °C (Kushner et al., 1977; Champney, 1979). For some of these mutants, the ribosome has been identified as the source of the temperature-sensitive protein synthesis activity.

In order to quantitate the effect of temperature on the ribosomal activity and to identify the ribosomal component responsible for the temperature effect, we have conducted a

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series of kinetic assays at various temperatures, with the source of the ribosomes (control or mutant strains) as the only variable. Using phage MS2 RNA as a messenger and measuring the rates of ribosome-promoted protein synthesis, we have compared the effect of temperature on this process. We present data which show the temperature lability of the ribosomes and subunits from the mutants examined and which define the increased activation energy required to drive protein synthesis with mutationally altered ribosomes. These studies will be useful in relating the observed functional defects to structural alterations in various ribosomal components.

Materials and Methods

Materials. tRNA, polyuridylic acid, and pyruvate kinase (EC 2.7.1.40) were purchased from Sigma Chemical Co. Calcium leucovorin was from Lederle Labs. [^{14}C]Valine (250 mCi/mmol), [^{14}C]phenylalanine (460 mCi/mmol), and [^{3}H]uracil (40 Ci/mmol) were from Schwarz/Mann. Other biochemicals and chemicals used were of analyzed reagent grade.

Isolation of Protein Synthesis Components. The wild type control and temperature-sensitive mutant strains of *E. coli* used as the source of ribosomes in these assays have been described previously (Kushner et al., 1977; Champney, 1979). The ribosomes were isolated from cells grown at 30 °C and were washed once with 1 M NH_4Cl. Ribosomal subunits were separated by sucrose gradient centrifugation as previously described (Kushner et al., 1977). Ribosome-free S100 proteins were further purified by passage through a DEAE-cellulose column as described by Traub et al. (1971). Crude initiation factors derived from the first ribosomal wash were also isolated as detailed by Traub et al. (1971). Bacteriophage MS2 was grown on *E. coli* strain D10 (Gesteland, 1966) and purified from the lysate by CsCl gradient centrifugation as described by Nathans (1968). When required, the phage RNA was labeled by adding [^{3}H]uracil (1 μCi/mL) to the cell culture 5 min after infection. MS2 RNA was isolated by phenol extraction of the purified phage. The ribosomes, subunits, and S100 proteins were stored in R buffer (10 mM Tris-HCl,[1] pH 7.6, 10 mM magnesium acetate, 50 mM NH_4Cl, and 6 mM mercaptoethanol) at −70 °C. The initiation factors were stored as described in R buffer containing 0.25 M NH_4Cl.

In Vitro Protein Synthesis Assay. Cell-free protein synthesis programmed by phage MS2 RNA was conducted at seven different temperatures in a 50-μL reaction mixture containing the following: 50 mM Tris-HCl, pH 7.6, 10 mM magnesium acetate, 50 mM NH_4Cl, 6 mM dithiothreitol, 2 mM ATP, 1 mM GTP, 5 mM phosphoenolpyruvate, 25 μg of tRNA, 4.4 μg of calcium leucovorin (5-formyltetrahydrofolic acid), 0.1 mM each of 19 amino acids, and 0.25 μCi (0.002 mM) of [^{14}C]valine. The reaction mixture was supplemented with the following to promote the formation of MS2 RNA specific proteins: 1 μg of pyruvate kinase, 12 μg of S100 proteins, 19 μg of initiation factors, 1.67 A_{260} units of MS2 RNA (67 μg; Strauss & Sinsheimer, 1963), and 1.0 A_{260} unit (69 μg; Kurland, 1966) of control or mutant 70S ribosomes. Each of the preceding components was present in amounts which gave an optimum incorporation of [^{14}C]valine. After the appropriate incubation period, the reaction was terminated by the addition of 2 mL of 5% trichloroacetic acid and the samples were heated at 95 °C for 20 min, cooled, and filtered through GF/A filters. The precipitable [^{14}C]valine radioactivity was measured in a 0.25% PPO–toluene solution in a liquid scintillation counter with a ^{14}C efficiency of 80%. Under these conditions the specific activity was 460 cpm/pmol of [^{14}C]valine.

Kinetic Assays. These were conducted by removing 10-μL aliquots from the incubation mixture at various times (up to 30 min) and measuring the amount of isotope incorporated as a function of time at a particular incubation temperature.

Inactivation Assays. The inactivation of 70S ribosomes at 44 °C was performed by incubating 6 A_{260} units of 70S ribosomes in 60 μL of R buffer. At various times (up to 2 h) samples of 1 A_{260} unit (10 μL) were removed and held on ice until the end of the preincubation. The remaining protein synthesis assay components were then added to each sample, and the total incorporation was determined after 30 min at 40 °C.

Subunit Mixing Assays. Assays with mixtures of control and mutant 50S and 30S subunits were conducted at 44 °C for 30 min. In this case, 0.6 A_{260} unit of 50S and 0.3 A_{260} unit of 30S subunits were mixed and assayed for the net incorporation of [^{14}C]valine. All four combinations of mutant and control subunits were assayed simultaneously, and the incorporation measured was corrected for that found in assays with only one subunit present (to measure the extent of subunit contamination). These assays were conducted with the reaction mixture described above, except that 4 μg of S100 proteins and 56 μg of initiation factors gave the optimum incorporation under these conditions.

^{3}H-Labeled MS2 RNA Binding Assay. Binding of radioactive phage MS2 RNA to 70S ribosomes was conducted by mixing 1.7–2.0 A_{260} units of ribosomes with 4.75 μg of initiation factors and 1 A_{260} unit of [^{3}H]uracil-labeled MS2 RNA (15 615 cpm/A_{260}) in 100 μL of R buffer. The samples were incubated for 10 min at 44 °C and then diluted with 1 mL of cold R buffer and immediately filtered through washed 0.45-μm Millipore filters. The filters were washed twice with 4 mL of cold R buffer and then dried and counted as described above. For some assays, the 70S ribosomes were incubated alone at 44 °C for 30 min before the MS2 RNA binding assay was conducted.

[^{14}C]Phenylalanine tRNA Binding Assay. Commercial tRNA was purified by phenol extraction, deacylated by incubation at pH 8.8, and charged with [^{14}C]phenylalanine (0.2 μCi) by incubation with 100 μg of DE-S100 in a 100-μL reaction mixture following the conditions of Kurland (1966) and Traub et al. (1971). The purified phenylalanine-charged tRNA preparation had a specific activity of 20–25 pmol of phenylalanine per A_{260} unit of tRNA (800 cpm/pmol of Phe-charged tRNA).

The polyuridylic acid dependent binding of [^{14}C]Phe-charged tRNA to 70S ribosomes was conducted by incubation at 44 °C for 30 min. The binding mixtures included 1 A_{260} unit of 70S particles with 40 μg of polyuridylic acid and 0.5 A_{260} unit of [^{14}C]phenylalanine-charged tRNA [(1.6–2.0) × 10^4 cpm/A_{260}] in a final volume of 50 μL of R buffer. After incubation, the samples were diluted with 1 mL of cold R buffer, filtered, washed, and counted as described for MS2 RNA assays. Some experiments included a 30-min preincubation of the ribosomes at 44 °C before the binding assay.

Ribosomal Subunit Reassociation Assay. The 70S ribosomes (2 A_{260} units) in R buffer were diluted about 25-fold into 100 μL of S buffer (R buffer containing only 0.5 mM magnesium acetate). After 20 min at 44 °C, 1 M magnesium acetate was added to give a final concentration of 20 mM and the incubation was continued for an additional 10 min. The ribosomes were then layered on a 5-mL 5–20% sucrose gra-

[1] Abbreviations used: A_{260}, absorbance at 260 nm of a 1-mL solution in a 1-cm light path; Tris-HCl, tris(hydroxymethyl)aminomethane hydrochloride.

dient of R buffer containing 30 mM magnesium acetate and centrifuged in an SW 50.1 rotor for 135 min at 35 000 rpm. The gradients were fractionated through an ISCO flow cell, and the 254-nm absorbance was recorded. The areas of the subunit and 70S peaks were measured on the recorder tracing, and the percent reassociation was calculated as [area 70S peak/(area 30S + 50S + 70S peaks)]100.

Polyacrylamide Gel Analysis of [^{14}C]Valine Proteins. Control ribosomes were used in duplicate protein synthesis assays at each of the seven temperatures used in the kinetic assays. After a 30-min incubation, the duplicate samples were combined and 50 μg of MS2 proteins, purified from the phage as described by Nathans (1968), was added to each. The samples were made 0.02 M in EDTA and were incubated for 30 min at 37 °C in the presence of 5 μg of pancreatic RNase and 0.5 μg of T_1 RNase. After the incubation, 1 mL of 10% trichloroacetic acid was added to each sample and the precipitates were removed by centrifugation. The pellets were washed once with acetone and dried under vacuum. The dried proteins were dissolved in 80 μL of sodium dodecyl sulfate ($NaDodSO_4$) sample buffer, heated at 100 °C for 4 min, cooled, and loaded onto the slots of a 10% polyacrylamide slab gel of the composition described by Weber & Osborn (1969). Molecular weight standards (20 μg each of bovine serum albumin, peroxidase, trypsin, lysozyme, and cytochrome *c*) were loaded onto adjacent wells of the gel. The gels were run for 8 h at 50 V. The proteins were stained with Coomassie Blue, and each sample lane was cut into 20 5-mm slices. The gel slices were hydrolyzed in 0.5 mL of 30% H_2O_2 at 60 °C, and the radioactivity present was measured in a toluene–Triton X-100 scintillation-counting fluid (Schaup et al., 1970).

Results

These studies were initiated to investigate the basis of the temperature-sensitive protein synthesis observed in a collection of mutants of *E. coli* which we have isolated (Kushner et al., 1977; Champney, 1979). Preliminary studies had indicated that the ribosomes, but not the S100 protein fraction, from these mutant strains displayed a heat-sensitive activity in cell-free protein synthesis assays. The details of the sensitivity were initially examined by observing the rates of MS2 RNA directed protein synthesis with kinetic assays at seven different temperatures. Ribosomes isolated from a control strain, able to grow at 44 °C, and from seven conditionally lethal mutants were used in the kinetic experiments. The S100 proteins, initiation factors, and other components of the in vitro reaction mixture were derived from the control strain, so that the source of the ribosomes and the temperature were the only variables in the assays.

Phage MS2 RNA is known to direct the synthesis of three proteins in an in vitro translation system (Sugiyama & Nakada, 1968; Lodish, 1975). The phage coat protein represents the majority of the proteins formed, often representing more than 70% of the total product (Nathans et al., 1962; Lodish, 1968). Before the kinetic studies were attempted, we felt that it was important to know if the amount of the major protein product formed was related to the assay temperature. By use of control 70S ribosomes, the amount of [^{14}C]valine incorporated into the coat protein was determined in assays conducted at each of seven temperatures. The coat protein formed in vitro was separated from the other proteins by gel electrophoresis and its [^{14}C]valine content determined. As the assay temperature increased from 30 to 47 °C, the relative amount of coat protein made increased to a maximum at 40 °C and declined above that temperature (Table I). The amount of isotope in the coat protein increased from 36% at 30 °C to 54% of the total amount incorporated at the temperature optimum, indicating the predominance of the formation of this protein. Total protein synthesis was also maximum at 40 °C (Table I), as was the rate of protein synthesis with the control ribosomes (see below).

Table I: MS2 Coat Protein Synthesis as a Function of Incubation Temperature

temp (°C)	total incorpn[a]	coat protein[b]	% of total
30	16.0	5.8	36.5
34	14.9	6.5	43.2
37	26.8	12.6	47.1
40	35.3	18.9	53.8
42	29.6	14.7	49.9
44	32.7	15.0	45.9
47	22.6	10.3	45.4

[a] Total picomoles of [^{14}C]valine incorporated in duplicate assays after 30 min at the indicated temperature, under the assay conditions described under Materials and Methods. [b] Total picomoles of [^{14}C]valine present in the region of the polyacrylamide gel containing the MS2 coat protein. Radioactivity present in the gel was determined as described under Materials and Methods.

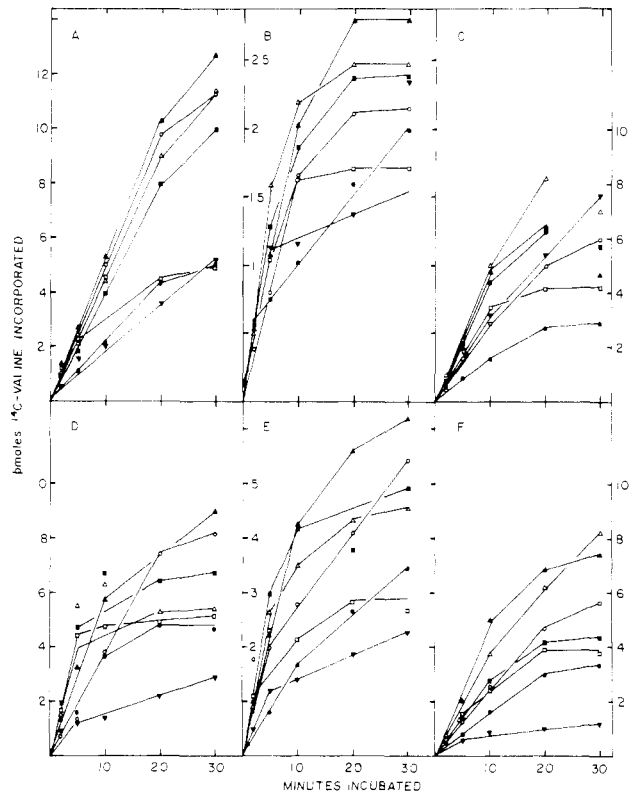

FIGURE 1: Kinetics of in vitro protein synthesis at different temperatures. The rates of MS2 RNA directed [^{14}C]valine incorporation were examined at seven temperatures by using ribosomes from a control and five temperature-sensitive mutant strains. The assays were conducted as described under Materials and Methods at 30 (▼), 34 (●), 37 (○), 40 (▲), 42 (△), 44 (■), and 47 (□) °C. Assays were performed with ribosomes isolated from strains (A) SK901 (control), (B) SK911, (C) SK1029, (D) SK1154, (E) SK1155, and (F) SK1156.

The kinetic assays were conducted under conditions where the ribosomal activity was the limiting factor in the amount of amino acid incorporated. For purposes of analysis, protein synthesis was considered to be an irreversible reaction, so that initial velocities of amino acid incorporation could be used directly to calculate rate constants at different temperatures.

The rates of [^{14}C]valine incorporation into MS2 proteins as a function of time at seven different incubation temperatures are illustrated in Figure 1 for assays using ribosomes from the

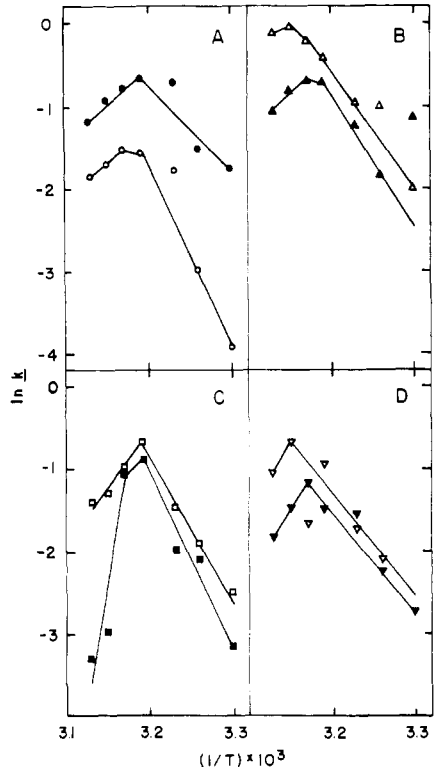

FIGURE 2: Arrhenius plots of protein synthesis rates as a function of assay temperature. The natural logarithm of the rate constant (k) for protein synthesis is given as a function of the reciprocal temperature (K) for the assays illustrated in Figure 1. (A) SK901 (●) and SK911 (○); (B) SK1029 (▲) and SK1154 (△); (C) SK1155 (■) and SK1156 (□); (D) SK1219 (▼) and SK1221 (▽).

control strain (SK901) and from five different temperature-sensitive mutants. Kinetic assays (not shown) were also performed with ribosomes from two additional mutants, SK1219 and SK1221. The temperature range was 30–47 °C. The initial velocities of protein synthesis, picomoles of valine incorporated per minute, were calculated for the assay conducted at each temperature for each ribosome preparation. The rates were determined from a least-squares best-fit line to the initial data points. The reaction rates increased as a function of temperature up to a maximum of 40 or 42 °C for the different ribosome sources. From these data an Arrhenius plot was constructed by graphing the natural logarithm of the rate constant (k) vs. reciprocal temperature (K) for protein synthesis employing the ribosomes from the control and each mutant strain. These graphs are shown in Figure 2. The slopes of the Arrhenius plots, from 30 °C to the maximum temperature, were used to calculate the activation energy, E_a (Mahler & Cordes, 1966). The activation energy for protein synthesis over this temperature range is listed for each strain in Table II. For the control, the value was 12 kcal/mol. With ribosomes from each of the mutant strains an increased activation energy was required to drive protein synthesis over this temperature interval (Table II). For the different mutants the increase was from 3 (SK1219) to 16 (SK911) kcal/mol. This increase in the required activation energy reflects a reduced efficiency in protein synthesis inherent in the ribosomes derived from the various temperature-sensitive mutants. Although the activity of the mutant ribosomes in protein synthesis was reduced relative to the control, the net free-energy change at 40 °C ($\Delta G^{\ddagger}$), calculated from the initial velocity at this temperature [$k = k_B T/h \exp(\Delta G^{\ddagger}/RT)$], was essentially the same, ~21 kcal/mol, regardless of the source of the ribosomes (Table II). This indicates that the final state of the overall

Table II: Arrhenius Activation Energy and Free-Energy Change for Protein Synthesis[a]

strain	E_a (kcal/mol)	$\Delta G^{\ddagger}$ (kcal/mol)
SK901	12	21.2
SK911	28	21.8
SK1029	21	21.3
SK1154	17	21.1
SK1155	23	21.4
SK1156	19	21.2
SK1219	15	21.8
SK1221	16	21.4

[a] The Arrhenius activation energy was calculated by using a least-squares method for the slopes of the lines in Figure 2, over the range of 30–40 °C. $E_a = -(\text{slope})R$. $\Delta G^{\ddagger}$ was calculated from the protein synthesis rate at 40 °C (Figure 1), using the equation $k = k_B T/h \exp(\Delta G^{\ddagger}/RT)$.

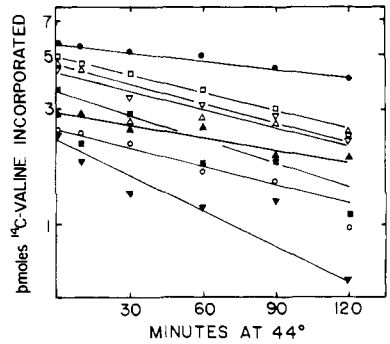

FIGURE 3: Inactivation rates at 44 °C for 70S ribosomes. Ribosomes from the control and mutant strains were preincubated at 44 °C and assayed for activity at 40 °C as described under Materials and Methods. The inactivation was conducted with 70S ribosomes from strains SK901 (●), SK911 (○), SK1029 (▲), SK1154 (△), SK1155 (■), SK1156 (□), SK1219 (▼), and SK1221 (▽).

process, i.e., the formation of MS2 proteins, was unaffected by the ribosomal source and that the differences in efficiencies reflect differences in rates and amounts of protein formation.

Above the maximum temperature, the Arrhenius plots have a positive slope (Figure 2). This reduction in protein synthesis capacity is related to the inactivating effect of high temperature on the ribosomes and other components necessary for protein synthesis. An inspection of Figure 2 reveals that except for SK1154, the slopes of the Arrhenius plots are steeper for protein synthesis with each of the mutants than for SK901. This indicates that less energy was required to inactivate the process of protein synthesis above the temperature optimum and is another indication of the temperature sensitivity of protein synthesis displayed by the ribosomes from these mutant strains.

The reduced activity of these ribosomes in protein synthesis at several temperatures suggested that the ribosomes from the mutants might be more temperature labile during incubation at the elevated temperatures. We examined the loss of protein synthesis activity after preincubation of the 70S ribosomes from each strain for different times at 44 °C. This temperature was chosen because all of the mutants are completely growth inhibited at this temperature (Champney, 1979). The inactivation kinetics for the 70S ribosomes are shown in Figure 3. The ribosomes from each of the mutant strains were more sensitive to the 44 °C preincubation than were the control ribosomes. A comparison of the half-time for inactivation ($t_{1/2}$ = time required for 50% loss of activity) is given in Table III and shows that the value was reduced from 250 min for SK901 to between 188 and 73 min for the ribosomes from the various mutants examined.

Table III: Inactivation Rates for Ribosomes from Control and Mutant Strains[a]

strain	$t_{1/2}$ (min)
SK901	250
SK911	92
SK1029	188
SK1154	110
SK1155	87
SK1156	112
SK1219	73
SK1221	123

[a] $t_{1/2}$, the time required for 50% loss of activity during a preincubation at 44 °C, was calculated from the slopes of inactivation curves like those in Figure 3 for assays conducted with 70S ribosomes from the control and mutant strains, as detailed under Materials and Methods. The relation $t_{1/2} = -\ln 2/\text{slope}$ was used in the calculations with the slopes computed by the method of least squares.

A very good correlation was found between the velocity of protein synthesis at 44 °C and the rate of inactivation of the ribosomes at this temperature. By comparison of the relative rates of activity and inactivation of the control and mutant ribosomes, a coordinate reduction in function and stability was apparent (Figure 4). The ribosomes from the control strain and all of the mutants except SK1219 showed an approximate 1:1 relationship between relative activity and inactivation.

The findings from these inactivation experiments were extended by independent subunit mixing assays performed at 44 °C. Combinations of control and mutant 30S and 50S subunits were assayed to determine which control subunit could restore activity with the complementary mutant subunit. From the data presented in Table IV, it is clear that control 30S subunits were effective in restoring activity (1.5–6-fold increase) when incubated with 50S subunits from mutants SK911, SK1029, SK1154, SK1155, SK1219, and SK1221. Effects with control 50S subunits were observed with 30S subunits from SK911 and SK1156. Thus, both subunits were apparently temperature sensitive in SK911, the 50S subunit was affected in SK1156, and the remainder of the mutants had heat-labile 30S subunits.

To help identify the specific functional defects in protein synthesis associated with the ribosomes from these mutant strains, we have performed three assays for ribosome function at 44 °C. By comparison with the control, ribosomes from the mutant strains showed deficiencies in mRNA binding, in tRNA binding, or in subunit reassociation. Figure 5 shows typical results from the latter assay. The control subunits reassociated to give 71% 70S ribosomes after being heated at 44 °C for 30 min while reductions to 55, 44, and 42% were seen when the subunits were derived from strains SK1029, SK911, and SK1156, respectively. The results of these assays for each strain are given in Table V.

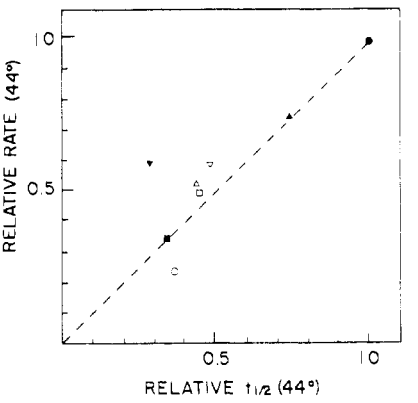

FIGURE 4: Correlation of protein synthesis rates and ribosomal inactivation rates at 44 °C. The rates of protein synthesis at 44 °C for the ribosomes from the seven mutant strains were calculated as a fraction of the rate of SK901 from data like that shown in Figure 1. Similarly, the relative half-life ($t_{1/2}$) of the ribosomes from the mutant strains was calculated as a fraction of the $t_{1/2}$ for SK901 from data like that in Figure 3. The relative rates of activity and inactivation are given for strains SK901 (●), SK911 (○), SK1029 (▲), SK1154 (△), SK1155 (■), SK1156 (□), SK1219 (▼), and SK 1221 (▽). The dashed line represents the slope expected for a 1:1 correlation between activity and inactivation.

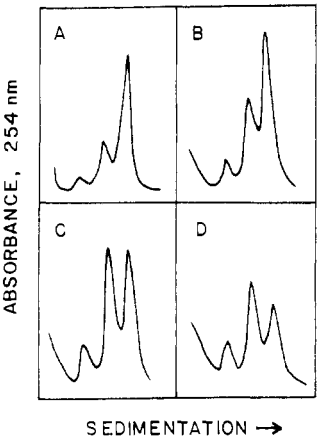

FIGURE 5: Sucrose gradient profiles of reassociated ribosomal subunits. The extent of ribosomal subunit reassociation at 44 °C was measured by examining the fraction of subunits and 70S ribosomes in sucrose gradients as detailed under Materials and Methods. Reassociation was examined with subunits from strains (A) SK901, (B) SK1029, (C) SK911, and (D) SK1156. The peaks in each gradient (from right to left) are 70, 50, and 30 S.

The association of mRNA and tRNA with 70S ribosomes from the different strains was tested at 44 °C, with and without a preincubation of the ribosomes. Substantial reductions in mRNA binding were observed with the ribosomes from strains SK911, SK1029, SK1219, and SK1221 (Table V). For ribosomes from the latter two strains, preincubation reduced the

Table IV: Mixed Ribosomal Subunit Assays at 44 °C[a]

strain	30S-C 50S-C	30S-M 50S-C	30S-C 50S-M	30S-M 50S-M	ts mutant subunit[b]	stimulation by control subunit
SK911	12.80	9.60	9.40	4.60	both	2.06
SK1029	10.30	3.15	6.15	2.89	30S	2.13
SK1154	9.45	4.17	16.30	4.03	30S	4.04
SK1155	11.75	2.11	5.65	2.97	30S	1.90
SK1156	11.84	6.29	1.96	1.10	50S	5.72
SK1219	11.37	1.06	4.76	0.85	30S	5.60
SK1221	8.70	3.66	6.67	4.60	30S	1.45

[a] The values given are picomoles of [^{14}C]valine incorporated in a 30-min assay at 44 °C with mixed ribosomal subunits as described under Materials and Methods. The source of the subunit is designated as control (SK901) 30S (30S-C) or 50S (50S-C) and mutant 30S (30S-M) or 50S (50S-M). The results are the average of duplicate assays with a difference between assays of ±5%. [b] ts = temperature sensitive.

Table V: Assays for Ribosome Functions at 44 °C

strain	% subunit reassocn[a] at 44 °C*	% mRNA binding[b] 44 °C	% mRNA binding[b] 44 °C*	% tRNA binding[c] 44 °C	% tRNA binding[c] 44 °C*
SK901	71	100	100	100	100
SK911	44	65	73	77	89
SK1029	55	64	66	41	36
SK1154	54	95	84	78	71
SK1155	53	78	78	69	71
SK1156	42	126	153	99	90
SK1219	69	99	72	69	68
SK1221	65	71	40	97	75

[a] The reassociation of the ribosomal subunits after incubation at 44 °C was measured by sucrose gradient centrifugation as described under Materials and Methods. Typical gradient profiles are shown in Figure 5. [b] Binding of ^{3}H-labeled MS2 RNA to 70S ribosomes was conducted as described under Materials and Methods at 44 °C without (44 °C) and with (44 °C*) a 30-min preincubation of the ribosomes. For SK901, 100% = 1850 (44 °C) and 1600 (44 °C*) cpm/A_{260}, respectively. [c] Binding of [^{14}C]-Phe-charged tRNA to 70S ribosomes was conducted as described under Materials and Methods at 44 °C without (44 °C) and with (44 °C*) a 30-min preincubation of the ribosomes. For SK901, 100% = 3485 (44 °C) and 5590 (44 °C*) cpm/A_{260}, respectively.

mRNA binding by ~30%. Six of the seven mutants also showed some reduction in tRNA binding, with the largest effect seen in SK1029 (Table V). Only the ribosomes from strain SK1156 demonstrated no significant impairment in these two binding assays.

Discussion

The purpose of these studies was to investigate the effect of temperature on ribosomal activity in protein synthesis. We hoped to be able to quantitate the degree of temperature sensitivity of the ribosomes from certain mutant strains of *E. coli* and to relate this to specific defects in protein synthesis in these strains.

Our initial experiments were conducted to define certain kinetic and thermodynamic parameters of normal protein synthesis using control ribosomes programmed with a natural mRNA. The amount of the major phage gene product formed as a function of translation temperature was determined to ask about the relative efficiency of the process. The results suggested a very good correlation between the amount of coat protein formed and the rates of in vitro synthesis as a function of temperature. With the control ribosomes, the rate and amount of specific protein synthesis were maximum at 40 °C. This indicated that an analysis of the rates of synthesis with the mutant ribosomes would be representative of net protein synthesis. Differences in mRNA secondary structure could account for the 40 °C optimum observed (Lodish, 1975).

It is known that only a fraction of purified ribosomes will be active when assayed in vitro (Zamir et al., 1974; Lodish, 1976). An important question is whether or not this active fraction of ribosomes can accurately translate mRNA into defined proteins. This question was answered experimentally, as the data in Table I indicate. A substantial amount of MS2 coat protein was formed by the ribosomes, and the amount made was a function of the incubation temperature. A second point relevant to the question of their efficiency is the extent of mRNA translation. From Table I, 50 pmol of MS2 RNA promoted the incorporation of 19 pmol of valine (at 40 °C) or 190 pmol of coat protein (valine = 10% of coat protein). Thus, each mRNA coat protein sequence was translated into four molecules of specific protein. This efficiency is identical with that reported by others (Nathans et al., 1969; Lodish, 1975). Furthermore, the extent of incorporation achieved in our assays in terms of picomoles of amino acid incorporated per picomole of ribosomes was similar to that reported by several other authors (Funatsu et al., 1972; Revel et al., 1973). Finally, the amount of ribosomes used in these assays was intentially kept low in order to make the incorporation dependent on the ribosome concentration so that control and mutant particles could be validly compared. The differences in rates and extent of amino acid incorporation observed with the ribosomes from the mutants at different temperatures *must* therefore be a direct effect of temperature on the activity of the ribosomes in the assay, particularly since all of the ribosomes were isolated by the same procedure and all of the other reaction components were identical in each case.

At the temperature optimum the free-energy change required to place amino acids into protein was 21.2 kcal/mol. By recognition of the complexities and reduced efficiency of cell-free protein synthesis (Tai et al., 1973), this value is still within the range expected for complex enzyme-catalyzed, sequential reactions (Mahler & Cordes, 1966; Spirin, 1978). For achievement of this change of state, an activation energy of 12 kcal/mol was calculated from the slope of the Arrhenius plot. This value represents the total energy required to drive the entire process, which includes the steps of tRNA charging and ribosome binding, initiation, elongation, and termination. Although the activation energy requirements for the individual steps in translation are unknown, it has been shown that the elongation steps of translocation and peptide bond formation require an activation energy of 5–6 kcal/mol (Pestka, 1969, 1970, 1972). Thus, about one-half of the total energy barrier to protein formation is provided by the mechanism of peptide bond formation. By contrast, the activation energy required for in vitro reassembly of the 30S ribosomal subunit was calculated to be ~38 kcal/mol (Traub & Nomura, 1969), a value more than 3 times that found for total in vitro protein synthesis under our conditions.

Above the temperature optimum, reduced rates of protein synthesis were observed. This reduction presumably reflects an opposition to the energy needed to drive protein synthesis at these elevated temperatures by the simultaneous thermal denaturation of components of the assay system. The control ribosomes showed a mild thermal lability, with a half-time for inactivation of 250 min at 44 °C. Other components of the reaction mixture are presumably more temperature sensitive and may lose activity at a greater rate.

The ribosomes from each temperature-sensitive mutant examined were less effective in the protein synthesis assays. The initial velocities were reduced at each temperature relative to the control ribosomes, and the slopes of the Arrhenius plots were correspondingly greater. An increased activation energy for protein synthesis was required in each case. Since the source of the ribosome was the only variable present, this increased energy requirement indicated a reduced efficiency in protein synthesis inherent in the ribosomes from the temperature-sensitive mutant strains. Although the efficiency of the reaction was reduced when these ribosomes were used, the process could be completed in every case. This is evidenced by the identity of the free-energy change at 40 °C for the reaction utilizing the different ribosomal preparations.

A careful examination of the reaction kinetics revealed that the extent of the reaction at elevated temperatures was reduced in the assays with the ribosomes from the mutant strains. This observation suggested a progressive inactivating effect of temperature on the activity of these mutant ribosomes. This inference was confirmed by showing that the ribosomes from

the mutant were inactivated more rapidly during an incubation at 44 °C, compared to control ribosomes. All of the ribosomes tested exhibited a first-order decay with time, suggesting that a single-sensitive component was being affected by the preincubation. By comparison of the ribosomes from the control strain and the different mutants, a direct correlation between the velocity of protein synthesis and the loss of activity at 44 °C was observed. This suggests an effect of temperature on a component of the ribosome directly involved in protein synthesis, i.e., a functional consequence.

A direct indication of the affected ribosomal subunit was provided by the results from the subunit mixing assays. These were designed as in vitro complementation assays at 44 °C with control and mutant ribosomal subunits. Control 30S subunits stimulated the protein synthesis activity with mutant 50S subunits from five strains. This indicated that the 30S subunit was the temperature-sensitive component in the ribosome from these mutants. The 50S subunit of strain SK1156 was identified as being temperature sensitive by the assay, and both subunits of SK911 showed some stimulation in the presence of the complementary control subunit. An examination of the RNAs and proteins from the subunits of these strains is currently being conducted to look for structural alterations in these macromolecules.

Assays for specific ribosomal functions indicated that ribosomal subunits from five of the seven strains tested were deficient to some extent in the ability to reassociate at 44 °C. The largest effects were observed in strains SK911 and SK1156, the two identified as having temperature sensitivity conferred by the 50S subunit. These strains showed the same reduction in tRNA binding activity after preincubation, but only SK911, with an indicated 30S subunit defect, was additionally reduced in mRNA binding. The ribosomes from these two strains behaved similarly in terms of their relative activity and inactivation rate at 44 °C. These similarities suggest a common effect through a 50S subunit structural component.

The remaining mutants were identified as having temperature sensitivity residing only in the 30S subunit from the mixing assays. Ribosomes from SK1154 and SK1155 gave similar levels of reduced activity in all three assays for function. They also gave a similar functional response, as reflected in the correlation between activity and inactivation at 44 °C. Finally, the ribosomes from SK1219 and SK1221 showed reduced activity in both tRNA and mRNA binding, with SK1221 showing the greatest mRNA binding defect among the strains examined. The ribosomes from SK1029 were maximally deficient in tRNA binding under these conditions. Thus, the temperature sensitivity of protein synthesis at 44 °C and the ribosomal inactivation at this temperature can be partially defined by specific defects in protein synthesis steps with the ribosomes from the mutant strains examined. We have not determined as yet whether these changes in activity are the consequence of single or multiple mutational alterations of ribosomal components.

Numerous examples have been given of the complex interactions characterizing the many proteins which belong to the bacterial ribosome (Apirion & Saltzman, 1974; Saltzman et al., 1974; Schlessinger, 1974). It is generally felt that most ribosomal activities are defined by groups of proteins acting cooperatively for a particular function (Stöffler & Wittmann, 1977). For this reason it is apparent that an alteration of any single ribosomal protein could either directly or indirectly affect a particular activity of the particle. In this regard it is significant that many of the activities assayed using the mutant ribosomes were not completely lost by extensive heating. Presumably, this represents a residual activity retained by the normal proteins in the presence of the altered macromolecule. It is also important to emphasize that in vivo the major effect of the ribosomal alteration may be on subunit assembly (Champney, 1979) or protein modification (Kushner et al., 1977) instead of protein synthesis activity per se.

The temperature lability of the ribosomes from the mutant strains suggests that structural alterations of the ribosomal macromolecules are present which affect the activity of the ribosomes in protein synthesis. For SK911, alterations of five 50S subunit proteins have been described (Kushner et al., 1977) and an alteration of 30S protein S10 has also been detected (W. S. Champney, unpublished experiments). These observations correlate well with the indications of a temperature effect on both subunits of the ribosomes from this mutant. Ribosomal subunit assembly at 44 °C was impaired in strains SK1029 and SK1221, and all of the mutants showed reduced rates of in vivo protein synthesis at 44 °C (Champney, 1979).

An important inference from these experiments is that the ribosomes from these mutants contain altered proteins (or RNA) which confer temperature sensitivity on a ribosomal subunit. The ribosomes can function during cell growth at low temperatures, but the efficiency of the process of protein synthesis is reduced, as evidenced by the increased activation energy requirement. At higher temperatures (above 40 °C) the mutant ribosomes showed a progressively slower activity with time, reflecting their inactivation at the restrictive temperatures. Presumably, the same thing occurs in vivo. This inhibition of activity (or defect in ribosome assembly), as the consequence of a temperature-labile component, eventually leads to cell death. The magnitude of the temperature effect for each mutant may depend on the specific macromolecule which has been affected, the rates of ribosome synthesis at the restrictive temperatures, and the ability of the preexisting ribosomes (formed at the permissive growth temperatures) to function at the restrictive temperatures.

We are presently working to define the nature of the structural alteration of these ribosomes in order to better relate the normal structure to the correct function of the ribosome in protein synthesis. A detailed examination of the ribosomal proteins or RNA may reveal important information about the altered state of these macromolecules which leads to temperature sensitivity.

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Direct Iodination of Specific Residues in Crystals of Yeast Formylatable Methionine-Accepting Transfer Ribonucleic Acid†

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ABSTRACT: Crystals of yeast formylatable methionine-accepting transfer ribonucleic acid (tRNA$_f^{Met}$) were iodinated by using a modification of Commerford's procedure [Commerford, S. L. (1971) *Biochemistry 10*, 1993]. Chromatographic analysis of nuclease digestion products showed that radioactive iodine binds covalently to the 5 position of three nucleotide residues: U_8, C_{73}, and C_{74}. These three iodine substitutions were assigned to three peaks in a difference Fourier synthesis comparing the iodinated derivative with native tRNA$_f^{Met}$. In this way the positions of U_8, C_{73}, and C_{74} were marked in the crystal structure of yeast tRNA$_f^{Met}$, providing guidepoints for the interpretation of a 4.5-Å electron density map.

The interpretation of electron density maps from X-ray diffraction studies of macromolecules is facilitated by isomorphous derivatives which mark specific residues with heavy atoms. One can combine X-ray diffraction and chemical sequencing methods to map the heavy-atom binding sites in the crystal onto their corresponding locations in the sequence, thus providing guidepoints for tracing the molecular backbone (Sigler et al., 1966; Dunill et al., 1966; Pasek et al., 1973; Rosa & Sigler, 1974; Schevitz et al., 1975). In certain cases, the markers can be used to establish the relative orientation and position of similar structures in different crystal forms (Matthews et al., 1968; Wyckoff et al., 1969; Sigler, 1970).

The heavy atom employed as a marker should satisfy the following criteria. (a) It should have an atomic number high enough to be readily located in a difference Fourier synthesis. (b) It should react covalently under mild conditions. (c) Its binding should not distort the crystal structure. (d) It should possess convenient radioisotopes to facilitate finding its position in the sequence. Iodine is a natural choice.

Commerford (1971) has shown that a mixture of KI and $TlCl_3$ will iodinate polynucleotides in solution with a strong preference for single-stranded pyrimidine residues. The reaction proceeds in two steps (Figure 1): first, a rapid uptake of iodine to form a labile intermediate, followed by conversion to products or reversion to starting materials (Commerford, 1971; Anderson & Folk, 1976). Both poly(C) and poly(U) incorporate comparable amounts of iodine in the first step, but conversion to iodopyrimidine is much greater for poly(C) (Commerford, 1971). Schmidt et al. (1973) iodinated yeast tRNA$_f^{Met}$ in solution by this method and presented evidence for the iodination of three cytidines, two at the 3′-terminal C-C-A end and one in the anticodon loop. Recently, Batey & Brown (1977) iodinated several tRNA species and found that they could iodinate only C residues which were considered "exposed" by other criteria. We have employed a modification of Commerford's method to iodinate crystals of yeast tRNA$_f^{Met}$ directly. To facilitate the chemical analysis, we have used ^{125}I in all experiments. We have shown that there are three major sites to which iodine is covalently attached, namely, the 5 position of residues U_8, C_{73}, and C_{74}, and have assigned these covalently bound iodine atoms to three peaks in a difference

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