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Total reconstitution of active large ribosomal subunits of the thermoacidophilic archaebacterium *Sulfolobus solfataricus*

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ABSTRACT

The large ribosomal subunit of the extremely thermoacidophilic archaebacterium Sulfolobus solfataricus has been reconstituted from the completely dissociated RNA and proteins by a two-step incubation procedure at high temperatures. Successful reconstitution requires a preliminary incubation of the ribosomal components for 45 min at 65°C, followed by a second heat-treatment at 80°C for 60 min. Structural reassembly depends upon high concentrations of K^+ (300-400 mM) and Mg^{2+}(20-40 mM) ions. In addition, complete recovery of subunit function stringently requires the presence of a polyamine, thermine (or spermine). The reconstituted archaebacterial subunits are essentially indistinguishable from the native ones by a number of structural and functional criteria.

INTRODUCTION

In vitro assembly of functionally active ribosomal subunits from the dissociated RNA and protein components is a powerful tool for the understanding of ribosome structure and function. Reconstitution techniques have been extensively employed to elucidate the roles of the various constituents of E.coli ribosomes in both assembly and function (1,2). Ribosome reconstitution has also been used to establish functional homologies between ribosomal components in distantly related eubacterial species (3-6).

Until now, however, the lack of ribosome reconstitution procedures in organisms other than eubacteria has prevented comparisons between different systems of in vitro self-assembly. It has also hindered a direct testing of the degree of evolutionary conservation of the various ribosomal components in the three primary kingdoms, the eubacteria, the archaebacteria and the eukaryotes.

In this paper, we report the total in vitro reconstitution

of the large ribosomal subunits of Sulfolobus solfataricus, an extremely thermophilic archaebacterium which grows in solfataric hot springs at 87°C and pH 3-4 (7). Native large subunits of Sulfolobus ribosomes resemble those of eubacteria in having a prokaryote-sized 23 S RNA and roughly 34 proteins (8,9). However, they differ from eubacterial 50 S particles in several important aspects. Sulfolobus 50 S particles are appreciably heavier than those of eubacteria owing to a greater average mass of their subunit proteins (9). Three-dimensionally, they resemble more closely eukaryotic than eubacterial large ribosomal subunits (10). Finally, Sulfolobus 50 S particles are unique in being essentially insensitive to most antibiotics which, in eubacteria and eukaryotes, inhibit protein synthesis by interacting with the large ribosomal subunit (11).

The reconstitution system described in the present report provides a basis for investigating the ribosome assembly sequence in an archaebacterium and to determine the roles of the individual ribosomal constituents in both assembly and function. In addition, the method discloses the possibility of exploring the degree of structural and functional relatedness between components of eubacterial and archaebacterial ribosomes.

MATERIALS AND METHODS

Preparation of crude cell extracts

Sulfolobus solfataricus (DSM 1616) cells were grown as described (12) and harvested in the early phase of exponential growth (A_{660}=0.6). The cells were disrupted by grinding with twice their wet weight of Allumina powder while gradually adding 2.5 volumes of buffer A (20 mM Tris-HCl, pH 7.4, 10 mM Mg acetate, 50 mM NH_4Cl, 1.0 mM dithiotreitol) containing 2.5 µg/ml RNAse free-DNAse. Allumina and cell debris were removed by two low-speed centrifugations (30,000 x g for 30 min). The resultant supernatant was centrifuged in a Beckman Ti 50.2 rotor at 38,000 rev/min and 4°C for 20 min and the upper four-fifths of the final extract were used for RNA or ribosome isolation.

Preparation of whole-cell RNA

The cell extracts were adjusted to A_{260}=200 with buffer A and supplemented with 0.2 volumes of 10% (w/v) sodium dodecylsulpha-

te,0.4 volumes of 2%(w/v) bentonite and 1.0 vol of SCE buffer (150 mM NaCl,15 mM Na citrate,10 mM EDTA,disodium salt, pH 7.0). An equal volume of SCE-saturated phenol was added, and the mixture was shaken for 3 min at 4°C. The resultant aqueous phase was removed and extracted three more times with phenol in the presence of 0.4% (final conc) bentonite before RNA was precipitated by using 2.5 vol of ethanol.The RNA was dissolved in SCE buffer and ethanol precipitation was repeated twice. The last RNA pellet was resuspended in 10 mM Tris-HCl,pH 7.0,4.0 mM Mg acetate, at A_{260}=200, dialysed against the same buffer for 48 h, and stored in small samples at -25°C. The intactness of ribosomal RNA was checked by gel electrophoresis as described in (13).

Preparation of ribosomes and ribosomal subunits

The cell extracts were centrifuged in a Beckman Ti 50.2 rotor at 45,000 rev/min and 4°C for 2 h.The crude ribosomes were resuspended in buffer B (20 mM Tris-HCl,pH 7.2,40 mM NH_4Cl, 10 mM Mg acetate,2.0 mM dithiothreitol) and layered on a 7.0 ml cushion of 0.5 M sucrose in 20 mM Tris-HCl,pH 7.2, 500 mM NH_4Cl, 10 mM Mg acetate, 2.0 mM dithiothreitol. Ribosomes were pelleted by centrifugation in a Ti 50.2 rotor at 45,000 rev/min and 4°C for 5 h and resuspended in buffer B.

Ribosomal subunits were produced by centrifuging the purified ribosomes in a Beckman Ti 15 zonal rotor on a hyperbolic, 6 cm, 7-38% (w/v) sucrose density gradient in buffer C (20 mM Tris-HCl, pH 7.2, 500 mM NH_4Cl, 5.0 mM Mg acetate, 2.0 mM dithiothreitol, 0.5 mM spermine).The ribosome sample (2000-3000 A_{260}) was applied in a linear,0-7.4%(w/v) sucrose gradient (100 ml) and overlaid with 600 ml of buffer C. The gradient was centrifuged for 15 h at 4°C and 24,000 rev/min and was displaced with a 45% (w/v) sucrose solution. The subunits were collected by centrifugation in a Beckman Ti 50.2 rotor at 50,000 rev/min and 4°C for 20 h and dissolved in buffer B containing 50% glycerol, at A_{260}=700-800.

Preparation of total large subunit proteins (TP 50)

Total large subunit proteins were prepared according to Nierhaus and Dohme (14) with minor modifications. Aliquots (700-800 A_{260} units) of the 50 S subunit suspension were adjusted to 1.0 M Mg acetate,100 mM dithiothreitol and 66% (v/v) acetic acid. The solutions

were stirred for 60-90 min at 4°C and centrifuged at 10,000 rev/min. The resultant supernatant was supplemented with 5 volumes of acetone and allowed to stand at -20°C for 3 h. The precipitated proteins were collected by centrifugation at 10,000 rev/min for 10 min, liophylized to eliminate acetone, and dissolved in 2.4 ml of 6.0 M urea containing 20 mM Tris-HCl, pH 7.0, 20 mM Mg acetate, 400 mM KCl, 4.0 mM dithiotreitol. The solutions were then dialysed for 24 h against the latter solvent lacking urea, clarified by centrifugation at 5,000 rev/min for 5 min, and stored in small samples at -80°C. Protein concentrations are expressed as A_{260} equivalent units, where one A_{260} equivalent unit is the amount of protein obtained from one A_{260} unit of 50 S subunits, assuming no losses during extraction.

Reconstitution procedure

The reconstitution mixtures contained (in 80 µl): 20 mM Tris-HCl, pH 6.8, 20 mM Mg acetate, 300 mM KCl, 10 mM thermine hydrochloride, 2.0 mM dithiotreitol, 1.0 A_{260} unit whole-cell RNA (corresponding to approx. 0.5 A_{260} units 23 S RNA), 2.5 A_{260} equivalent units of TP 50. The mixtures were incubated at 65°C for 45 min. The Mg concentration was then raised to 40 mM by addition of 3.2 µl of 0.5 M Mg acetate and incubation was continued for 60 min at 80°C.

Characterization of the reconstitution products

To monitor physical reconstitution, reaction mixtures (80 µl) were layered onto 11.5 ml, 10-30% (w/v) sucrose density gradients containing 20 mM Tris-HCl, pH 7.0, 30 mM KCl, 20 mM Mg acetate, 2.0 mM dithiotreitol. The gradients were centrifuged in a Spinco SW 41 rotor at 35,000 rev/min and 10°C for 3 h. Effluent fractions corresponding to the 50 S peak of A_{260} were pooled and the particles therein collected by centrifugation in a Beckman Ti 50.2 rotor at 45,000 rev/min and 4°C for 24 h.

Thermal denaturation of ribosomal subunits was carried out by using a Beckman DU-8 recording spectrophotometer equipped with a thermal-melting program. The subunits were dissolved in 10 mM Tris-HCl, pH 7.0, 0.1 mM Mg acetate at A_{260}=0.5 and heated from 30°C to 105°C at a constant rate of 1°C/min, while changes in A_{260} were monitored continuously. Absorbance values were corrected for the thermal expansion of the solvent.

Two-dimensional polyacrylamide gel electrophoresis of ribosomal proteins extracted from native and reconstituted subunits was performed as described previously (9) and proteins were stained by the silver nitrate method (15).

Poly-U programmed polyphenylalanine synthesis

The standard mixture for the assay of subunit activity contained (in 180 µl): 20 mM Tris-HCl,pH 7.2, 20 mM Mg acetate, 33 mM KCl, 3.0 mM thermine (or spermine) hydrochloride, 2.0 mM dithiothreitol, 3.0 mM ATP, 2.0 mM GTP, 160 µg poly-U, 2 µl [^{14}C] phenylalanine (specific activity 800 counts/min/pmol), 5 pmol 30 S subunits, 20 µl of the reconstitution mixture (containing approx. 3 pmol of reconstituted 50 S particles) and an optimum amount of unfractionated proteins of the post-ribosomal supernatant fraction. The control assays contained 3.0 pmol of purified 50 S subunits. The mixtures were incubated for 40 min at 78°C. Hot-trichloroacetic acid precipitable radioactivity was determined as described in (16).

The effect of α-sarcin on poly-U directed polyphenylalanine synthesis was tested as described previously (11).

RESULTS AND DISCUSSION

RNA and protein preparation

Reconstitution of Sulfolobus 50 S subunits crucially depends upon the mode of isolation of both RNA and proteins. Ribosomal RNA extracted from either crude ribosomes or purified large subunits was found to contain several breaks masked by hydrogen bonds and consistently failed to promote ribosome assembly. Intact RNA, appropriately suited for reconstitution, was only obtained by phenol extraction of cell lysates and by using cells harvested during the early phase of exponential growth.Whole-cell RNA could be successfully used, 16 S RNA and low molecular weight RNAs having no detrimental effects on the assembly process.

Total proteins extracted from the 50 S particles (TP 50) were active in ribosome assembly only when the dialysis step required to remove urea from the protein solutions (see Methods) was carried out in the presence of no less than 300 mM KCl. As shown by Schulze and Nierhaus (17) a high monovalent cation concentration is required to prevent protein aggregation, which occurs in

Table 1. Optimal conditions for total reconstitution of active Sulfolobus large ribosomal subunits

	T (°C)	time (min)	K^+ (mM)	Mg^{2+} (mM)	thermine (mM)	pH
First step	65	45	300	20	10	6.8
Second step	80	60	300	40	10	6.8

low ionic strength solvents. By using proteins prepared in this way, the optimal stoichiometry for reconstitution was found to be approx. 2.5 A_{260} equivalent units of TP 50 per A_{260} unit of whole-cell RNA.

Reconstitution procedure and characterization of reconstituted particles

Total reconstitution of Sulfolobus large ribosomal subunits was achieved by incubating whole-cell RNA and TP 50 under the conditions specified in Table 1. Schematically, the procedure consists of two incubation steps at different temperatures (65°C and 80°C) in the presence of high concentrations of monovalent cations (300 mM), of relatively high Mg^{2+} concentrations (20 mM in the first step and 40 mM in the second) and of either thermine or spermine (10 mM). The efficiency and the accuracy of the

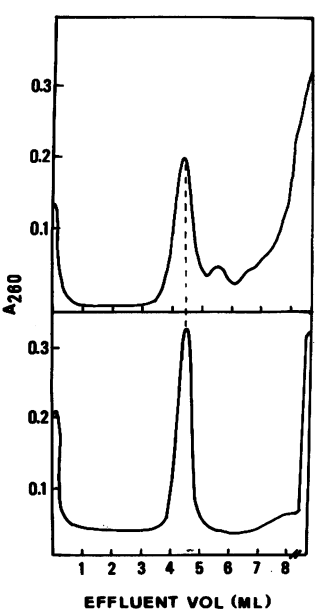

Fig.1. Sedimentation profiles of reconstituted (top) and native (bottom) Sulfolobus 50 S subunits on sucrose gradients.

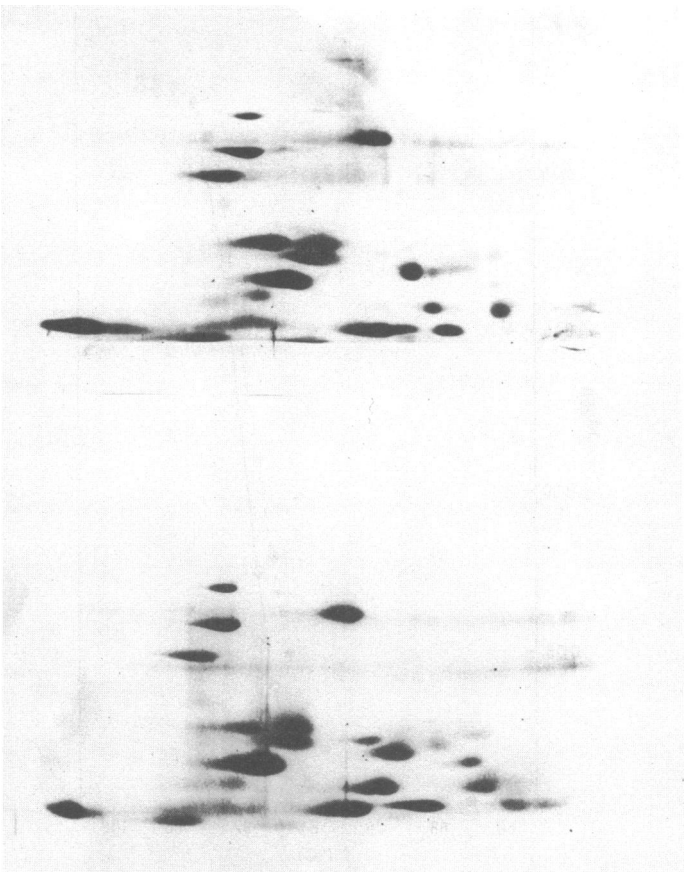

Fig.2. Gel-electrophoretic patterns of proteins extracted from reconstituted (bottom) and native (top) Sulfolobus 50 S subunits

assembly process were monitored by sedimentation analysis, gel electrophoresis of the subunit proteins, thermal 'melting' of the reconstituted particles and assay of synthetic activity in the poly-U system.

The sedimentation profiles in Fig.1 show that a sharp peak of A_{260}, having the same S value as native subunits, is formed from TP 50 and RNA under the conditions described in Table 1. The gel electrophoretic patterns of ribosomal proteins in Fig.2 show that the protein composition of the reconstituted particles is both

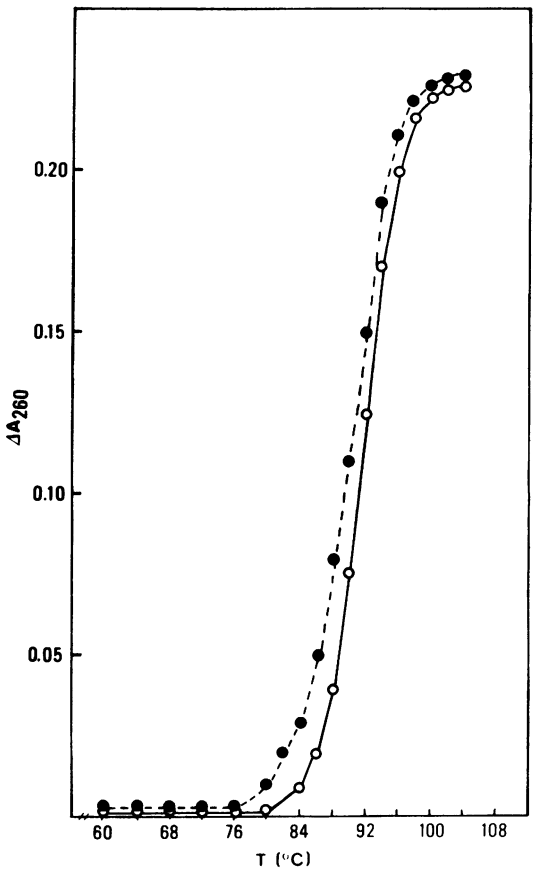

Fig.3. Thermal-denaturation profiles of Sulfolobus native (o) and reconstituted (●) 50 S subunits. ΔA_{260} is the increment in absorption at 260 nm of a ribosome solution of A_{260}=1.0 at 20°C

quantitatively and qualitatively superimposable on that of the native large subunits.

The physical integrity of the reassembly products is further documented by their thermal-melting behaviour. As Fig.3 shows, under optimum conditions for ribosome melting (18), reconstituted and native subunits display a same high melting temperature (T_m) of 90-92°C and an equally narrow width of the melting transition. Given the sensitivity of these parameters to even small unfolding of ribosome structure (19), such agreement as exists between the melting-hyperchromicity curves of native and recon-

Table 2. Polyphenylalanine synthesizing activity of Sulfolobus reconstituted large ribosomal subunits

	native 50 S +30 S	reconstituted 50 S + 30 S	30 S alone
counts/min/assay mix	25,350	18,430	340
mol phe/mcl 50 S	10.5	7.6	-
% activity	100	72.4	-

stituted subunits testifies the high degree of accuracy achieved by the assembly process.

The functional assays in Table 2 show that the reconstituted particles are appropriately designed for efficient peptide bond formation. The synthetic activity of the reconstitution products in the poly-U assay system (7-8 mol phenylalanine per mol 23 S RNA initially present in the incubation mixture) is no less than 70% that of an equimolar amount of native 50 S subunits. Given that a fraction of the 23 S RNA molecules may not participate in the assembly process, the latter figure is likely to represent a minimal estimate of functional restoration.

The conformational accuracy of the assembly products is also documented by the susceptibility of the reconstituted particles to α-sarcin, a polypeptide inhibitor of protein synthesis that inactivates both eukaryotic (20) and archaebacterial (21) ribosomes by cleaving the large subunit RNA at one specific site. Comparison of the inhibition curves in Fig.4 shows that the reconstituted particles are equally sensitive to α-sarcin as the native subunits. This is expected if the antibiotic binding site is properly reshaped during the in vitro reassembly process.

In the following paragraphs, we shall outline the elements that are of critical importance for obtaining total reconstitution of functional Sulfolobus large subunits. For the sake of clarity, the effects of temperature and ionic conditions on the assembly process are discussed separately, although this separation is somewhat artificial given the strict interdependence of all conditions involved.

Temperature dependence of subunit assembly

As might have been expected on biological grounds, the reconstitution of Sulfolobus ribosomes requires temperatures which

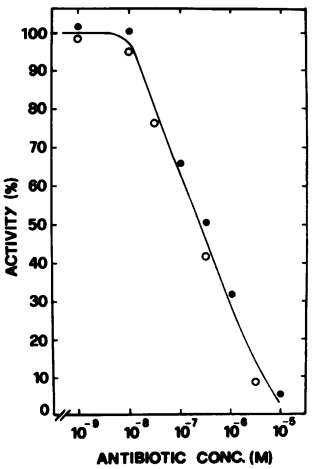

Fig.4. Effect of α-sarcin on the polyphenylalanine synthesizing activity of native (●) and reconstituted (o) subunits. Poly-U assays were performed as described in the Methods.

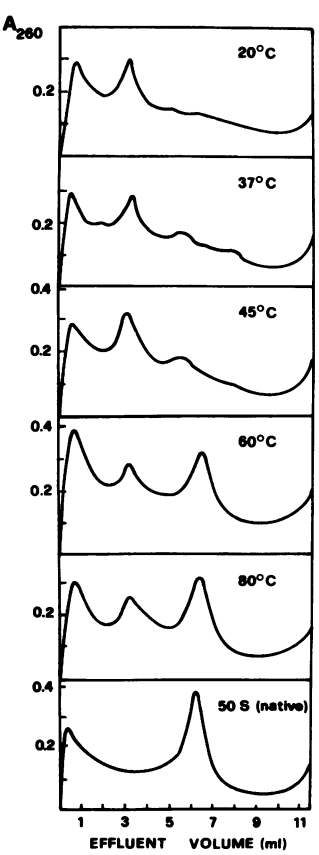

Fig.5.Temperature dependence of subunit reassembly. 2.5 A_{260} equivalent units of TP 50 and 1 A_{260} unit of whole-cell RNA were incubated at the indicated temperatures under the conditions specified in the text. After 60 min incubation the mixtures (80/ul) were centrifuged on sucrose gradients as described in the Methods section.

are considerably higher than those needed for the reconstitution of mesophile (E.coli) ribosomes (2). Physical reassembly of Sulfolobus 50 S particles requires no less than 60°C, while fine-tuning of subunit conformation demands temperatures close to the physiological optimum for cell growth.

The temperature dependence of physical assembly is illustrated in Fig.5, showing sucrose gradient patterns of reconstitution mixtures incubated for 60 min at the indicated temperatures in the presence of 20 mM Mg^{2+}, 10 mM thermine and 300 mM K^{+}. The results show that ribonucleoprotein complexes, sedimentally identical to native 50 S subunits, are only formed from TP 50 and RNA at temperatures equal to, or higher than, 60°C (Fig.5, d,e). Particles formed at either 60°C or 80°C contain a complete set of subunit proteins (not shown). Below 60°C, only minor amounts of slower-sedimenting, protein-deficient complexes are formed, regardless of incubation time and ionic conditions (Fig. 5 a,b,c). Apparently, a critical temperature of no less than 60°C is required to overcome conformational and/or energy barriers that prevent correct recognition between the assembly substrates. It is notable that 60°C is the lower limiting temperature for the growth of Sulfolobus cells (12).

Recovery of subunit function is related to temperature in a more complex fashion than physical assembly. This is illustrated in Fig.6, showing changes in the synthetic activity of the reconstitution mixtures during incubation at a fixed temperature (65°C or 80°C) or at two temperatures (first step at 65°C and second step at 80°C). To show the extent of functional restoration, the activity of the reconstitution products in the poly-U assay has been compared to that of an equivalent amount of native 50 S subunits taken as 100%. In the case of one-step reconstitution at 65°C, the synthetic activity of reconstituted and control particles was also assayed at 65°C, in order to rule out the possibility that functional activation of the assembly products might occur during incubation in the poly-U assay, which was routinely performed at 75-80°C.

The kinetics in Fig.6 show that no more than 40% the original activity is resumed upon incubating TP 50 and RNA at 65°C, whereas almost full activity (up to 70% that of the 50 S controls) is

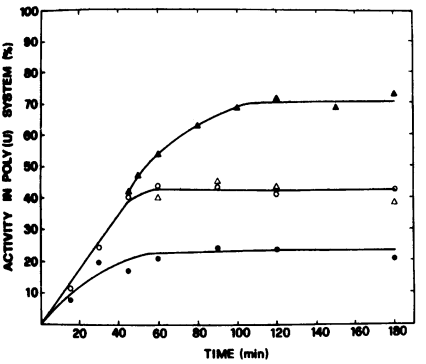

Fig.6.Kinetics of functional reactivation of reconstituted particles under different incubation conditions. Reaction mixtures (160 µl) containing 5 A_{260} equivalent units of TP 50 and 2 A_{260} units RNA were incubated as described below. At chosen time intervals 20 µl aliquots were withdrawn and assayed for poly-phe synthesizing activity (see Methods). (●)=single-step incubation at 80°C and either 20 mM or 40 mM Mg;(o)=single-step incubation at 65°C and 20 mM Mg;(▲)=incubation at 80°C and 40 mM Mg following a 45 min preheating at 65°C and 20 mM Mg;(△)=incubation at 80°C following 45 min at 65°C at a constant (20 mM) Mg conc.

recovered when the reconstitution mixtures incubated at 65°C for 45 min are subjected to a second heat-treatment at 80°C. Interestingly enough, when the incubation is started directly at 80°C, the degree of functional restoration is less than that observed for mixtures incubated at 65°C, even though the former temperature is closer to the physiological optimum for cell growth. This apparent paradox can be rationalized. Conceivably, free ribosomal components are more prone to hydrolytic attack than the assembly products, and hydrolases in the TP 50 preparations are more effective at close to physiological (80°C) than at lower (65°C) temperatures. Therefore, when TP 50 and RNA are directly incubated at 80°C, attendant hydrolytic action may result in the formation of functionally faulty particles.

In this context, the requirement for two incubation steps (65°C and 80°C) for full recovery of subunit activity can be envisioned as follows. A preliminary incubation at lower temperature is needed to allow the formation of complete-albeit poorly active-50 S particles under conditions that minimize hydrolitic damage of the assembly substrates. The subsequent incubation at

80°C acts by ultimately shaping the assembly products into a spatial design capable of efficiently interacting with other translational components. However, the temperature shift from 65°C to 80°C is effective only provided that the Mg concentration is raised from 20 mM (first step) to 40 mM (second step)(Fig.6).

It should be noticed that the mode of reconstitution of Sulfolobus large subunits resembles the classical protocol for reconstitution of E.coli 50 S particles (22,23) in requiring two heating steps having different temperature and Mg optima.Unlike the situation in E.coli,however,(23) in Sulfolobus the products of the first incubation already display significant activity in the poly-U assay; furthermore, no appreciable change in either the sedimentation velocity or the melting behaviour of the particles appears to accompany the increase in synthetic activity which occurs during the second heating step.

Ionic requirements

Unlike the situation in both mesophilic and thermophilic eubacteria (2,24),the functional reconstitution of Sulfolobus large ribosomal subunits is totally dependent upon the presence of polyamines throughout the entire assembly process. In the absence of polyamines, as will be discussed below, physical assembly of 50 S particles can take place, but the subunits thus formed are essentially inactive in polyphenylalanine synthesis.

As shown in Fig.7, three polyamines-thermine (1,11 diamino-4,8 diazaundecane),spermine (1,12 diamino-4,9 diazadodecane)and spermidine (1,8 diamino-4 azaoctane)-are effective in promoting reconstitution of TP 50 and RNA to synthetically active particles. In all cases, the optimal polyamine concentration is about 10 mM. However,thermine, the naturally occurring polyamine of Sulfolobus cells (25), is more effective than either spermine or spermidine.

In the presence of optimal polyamine concentrations,functional reconstitution is still dependent upon the presence of at least 20 mM Mg^{2+}ions. Experiments to reciprocally fine-tune the Mg and thermine concentrations during the initial incubation step at 65°C are summarized in Table 3. The results show that both the polyamine and the divalent cations are absolutely required for complete restoration of subunit function.In the absen-

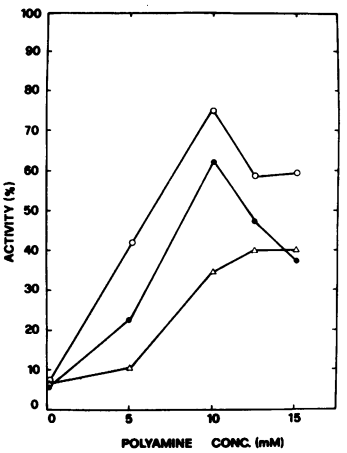

Fig.7. Polyamine dependence of subunit reconstitution. Reconstitution mixtures (see Methods) were incubated at 65°C for 45 min in the presence of varying concentrations of polyamines and of other components as specified in Table 1. The mixtures were then subjected to a second incubation as described in Table 1 except that the polyamine concentrations were kept constant throughout. (o)=thermine; (●)=spermine; (△)=spermidine

ce of thermine, physical reassembly of 50 S particles occurs only provided that 20 mM Mg,or higher,is present. Although particles formed under these conditions are sedimentally identical to native subunits and contain a complete set of TP 50,they display only negligible activity in the poly-U assay (Table 3). On the other hand, in the presence of an optimal (10 mM) thermine concentration, physical assembly is attained even at low (5 mM) Mg,

Table 3. Titration of optimal Mg and thermine concentration in the first incubation step

Thermine (mM)	Mg^{2+} (mM)	Peak of 50 S particles	Synthetic activity in poly-U assays
0	5	-	-
0	10	-	-
0	20	+	-
0	30	+	-
0	50	+	-
10	0	-	-
10	5	+	-
10	10	+	±
10	20	+	+
10	30	+	±
10	50	+	-

Synthetic activity indicated as ± corresponds to no more than 20% that of an equivalent amount of native 50 S subunits.After 45 min incubation at 65°C under the indicated conditions, the reconstitution mixtures were subjected to a second incubation at 80°C and 40 mM Mg for 60 min. In the mixtures containing 50 mM Mg this concentration was left unchanged during the second incubation.

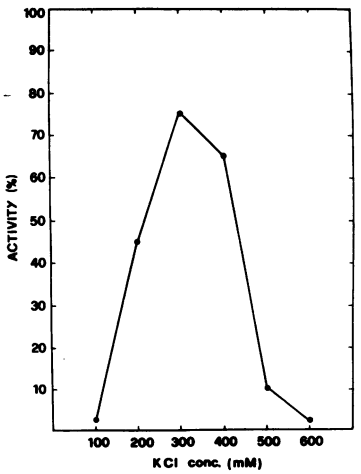

Fig.8. Monovalent cation dependence of subunit reconstitution. Reconstitution mixtures (see Methods) were incubated for 45 min at 65 °C in the presence of varying KCl concentrations and of other components as specified in Table 1. The mixtures were then subjected to a second incubation step as in Table 1, except that KCl was kept constant throughout.

but full synthetic activity is only resumed in the presence of 20 mM Mg. The results in Table 3 also show that impairment of subunit activity begins to occur above 30 mM Mg.

Fig.8 shows that a monovalent cation concentration of no less than 200 mM is required for reconstitution, optimal assembly occurring at 300-350 mM. Although both K^+ and NH_4^+ ions are equally effective, the former were routinely used because NH_4^+ ions strongly inhibit polyphenylalanine synthesis on Sulfolobus ribosomes, thus preventing measurements of functional activity by direct sampling of the reconstitution mixtures.

Undoubtedly,the most peculiar feature of the reconstitution of Sulfolobus ribosomes is the unique role played by polyamines in promoting restoration of particle function (see Fig.7).It is notable that spermine is also absolutely required for polyphenylalanine synthesis on Sulfolobus ribosomes (26);in this case, the polyamine appears to be essential for activating the peptidyltransferase center of the 50 S subunits (P.Londei,unpublished work).Taken together,these facts indicate that polyamines are essential structural components of Sulfolobus ribosomes,involved in properly shaping and stabilizing the quaternary packing of the 50 S subunits.It remains undecided whether the polyamine requirement of Sulfolobus ribosomes reflects adaptation to high temperatures of cell growth,or whether it constitutes a common phylogenetic distinction of sulphur-dependent archaebacteria.

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