The elongation factor 2 from the thermoacidophilic archaean Sulfolobus solfataricus (SsEF-2) binds \(^{3}H\)GDP at 1:1 molar ratio. The bound \(^{3}H\)GDP is displaced by GTP or its nonhydrolyzable analogue guanyl-5'-yl imidodiphosphate (Gpp(NH)p) but not by ATP, thus indicating that only the two guanosine nucleotides compete for the same binding site. The affinity of SsEF-2 for \(^{3}H\)GDP is higher than that for GTP and Gpp(NH)p. On the contrary, in the presence of ribosomes the affinity of SsEF-2 for GDP is lower than that for Gpp(NH)p. SsEF-2 is endowed with an intrinsic hardly detectable GTPase activity that is stimulated by ribosomes up to 2000-fold. The ribosome-stimulated SsEF-2 GTPase (GTPase') reaches a maximum at pH 7.8 and is not affected by ATP but is competitively inhibited by either GDP or Gpp(NH)p. Both \(K_m\) for \([\gamma-32P]GTP\) and \(k_{cat}\) of GTPase' increase with increasing temperature, and the highest catalytic efficiency is reached at 80°C. The ADP-ribosylation of SsEF-2 does not significantly affect either the binding of GDP and GTP or the kinetics of the GTPase'. A hypothesis on the stimulation by ribosome of SsEF-2 GTPase is proposed.

In the course of the protein synthesis the translocation of the peptidyl-tRNA from the A-site to the P-site of the ribosome is catalyzed in eubacteria by the elongation factor G and in eukarya and archaean by the elongation factor 2, both in the GTP-bound form. Following the interaction of the elongation factor-GTP complex with the ribosome, the GTP is hydrolyzed to GDP and P\(_i\), and the inactive GDP bound form of EF-2/EF-G leaves the ribosome. Therefore, the biological activity of EF-2/EF-G is regulated by the alternate binding of GDP and GTP (1–3).

In a previous study we have reported that the primary structure of the elongation factor 2 in the hyperthermophilic archaean Sulfolobus solfataricus (SsEF-2, formerly aEF-2) possesses the consensus sequences typical of the GTP-binding proteins (4). In the present report we show that SsEF-2 is able to bind guanosine nucleotides and to promote the hydrolysis of GTP in the presence of ribosomes as well. These findings support the hypothesis that the translocation step in the elongation of the polypeptide chain in archaean systems involves similar mechanisms as those described for eukarya and eubacteria (1, 5–7).

**EXPERIMENTAL PROCEDURES**

Materials—\(^{3}H\)GDP and \([\gamma-32P]GTP\) were purchased from Amer sham Corp.; unlabeled nucleotides and Gpp(NH)p were from Boehringer Mannheim. Diptheria toxin was obtained from Calbiochem. All of the reagents were of analytical grade.

The following buffers were used: buffer A, 25 mM Tris/HCl, pH 7.8, 10 mM NH\(_4\)Cl, 10 mM Mg(CH\(_3\)COO), buffer B, 50 mM Tris/HCl, pH 7.8, 2 mM Mg(CH\(_3\)COO), 50 mM KCl, 1 mM dithiothreitol; buffer C, 20 mM Tris/HCl, pH 7.8, 0.4 mM NH\(_4\)Cl, 10 mM Mg(CH\(_3\)COO), 0.05 mg/ml poly(U), 1 mM spermine, 1 mM ATP.

Methods—SsEF-2 was purified from S. solfataricus (ATCC 49255) as previously described (8) and stored at ~20°C in 20 mM Tris/HCl, pH 7.8, 10 mM MgCl\(_2\), 50% (v/v) glycerol. The purity of isolated SsEF-2 was assessed by SDS-polyacrylamide gel electrophoresis (9) or by reverse phase chromatography on a C\(_4\) column (Vydac) connected to a high performance liquid chromatography apparatus (Kontron).

Ribosomes were prepared according to the procedure described elsewhere (10) with an additional ultracentrifugation step at 100,000 x g at 20°C in buffer A supplemented with 18% sucrose and 500 mM NH\(_4\)Cl. The pellet was then suspended in buffer A containing 50% glycerol and stored at ~20°C. One A\(_{260}\) unit was taken to correspond to 25 pml of ribosomes.

Poly(U)-dependent poly(Phe) synthesis was performed as described previously (6).

The amount of the SsEF-2-[\(^{3}H\)GDP complex, formed upon incubation of \(^{3}H\)GDP with SsEF-2 in buffer B, was estimated by counting the radioactivity sticking on nitrocellulose filters (11), using a Packard Tri-Carb 1500 liquid scintillation analyzer. The apparent dissociation equilibrium constant, \(K_d\), of the SsEF-2-GDP complex was determined by titrating SsEF-2 with \(^{3}H\)GDP; the results were plotted according to the Scatchard equation (12), and both the \(K_d\) of the complex and the number of binding sites for \(^{3}H\)GDP on SsEF-2 were derived. The values of \(K_d\) of the SsEF-2-GTP and SsEF-2-Gpp(NH)p complexes were determined by competitive binding experiments between \(^{3}H\)GDP and GTP or Gpp(NH)p following the procedure described previously for SsEF-1\(_{a}\) (6); other experimental details are given in the legend to Fig. 1.

The ribosome-stimulated GTPase activity of SsEF-2 was measured in buffer C; ATP was maintained throughout even though it was essential only when the GTPase test was performed in the presence of partially purified ribosomes. The \([\gamma-32P]GTP\) hydrolyzed was estimated from the amount of \(^{32P}\) released, measured by the charcoal method (13); the reaction was followed kinetically at 60°C. Blanks run in the absence of SsEF-2 were subtracted. The rate of \([\gamma-32P]GTP\) breakdown was calculated from the slope of the linear kinetics of the hydrolytic reaction. The values of \(K_m\) and \(K_m'\) for \([\gamma-32P]GTP\), \(k_{cat}\) and \(K_i\), of GDP and Gpp(NH)p for GTPase' were determined as reported previously (13).

The ADP-ribosylation of SsEF-2 was performed as previously de-
RESULTS AND DISCUSSION

Binding of Guanosine Nucleotides to SsEF-2—The rate of [3H]GDP binding to SsEF-2 was very high, even at 0 °C (Fig. 1A), and it did not change appreciably in the pH range 5-9. Since SsEF-2-GTP is not retained on nitrocellulose filters, the binding of GDP to SsEF-2 was measured by competitive binding experiments in which the amount of the SsEF-2-[3H]GDP complex formed decreased at increasing concentrations of GDP (Fig. 1B). The nonhydrolyzable GDP analogue Gpp(NH)p was less effective, whereas ATP added up to 1000-fold molar excess over [3H]GDP was ineffective. The Scatchard plot shows that 1 mol of SsEF-2 binds 1 mol of [3H]GDP (Fig. 1C). At 60 °C the affinity of SsEF-2 for [3H]GDP (Kd = 1 μM) is 10-fold higher than that for GDP and 51-fold higher than that for Gpp(NH)p, thus resembling the behavior of EF-G/EF-2 from other sources (3, 15, 16).

SsEF-2 GTPase Activity in the Presence of Ribosomes—Ribosomes isolated from S. solfataricus possess a tightly associated GTPase activity. An additional ultracentrifugation step (see "Methods") reduced the ribosomal GTPase activity by 5-10-fold without affecting the ability of the ribosomes to support the in vitro poly(Phe) synthesis. When assayed in buffer C, purified SsEF-2 showed an intrinsically hardly detectable GTPase activity (less than 0.5 mol of [γ-32P]GTP hydrolyzed by 1 mol of SsEF-2 in 30 min at 60 °C). The addition of ribosomes stimulated a turnover SsEF-2 GTPase (Fig. 2A); its extent was maximum at 0.5 μM ribosome, which at higher concentrations produced inhibition (Fig. 2B), as already reported for the E. coli EF-G GTPase (18). The enzymatic [γ-32P]GTP breakdown followed linear kinetics and depended on the amount of SsEF-2 added. The possibility that SsEF-2 GTPase was due to a triphosphatase contaminant was ruled out by the following observations: (i) the sample of SsEF-2 used appeared homogeneous when analyzed by either SDS-polyacrylamide gel electrophoresis or by reverse phase chromatography on a C4 column; (ii) the typical properties of SsEF-2 (ability to be ADP-ribosylated by diphtheria toxin, GDP binding, and GTPase) co-purified with SsEF-2; (iii) the presence of a 10-fold molar excess of ATP over [γ-32P]GTP did not have any effect on the amount of GTPase released.

pH and Temperature Dependence, Catalytic Efficiency, and Energetic Aspects of GTPase—At 60 °C the pH optimum for the GTPase of SsEF-2 was in a narrow range, centered around pH 7.8, that was not substantially affected by the buffer used (Fig. 3).
In order to evaluate the effect of temperature on the rate and on the affinity of GTPase for $[\gamma-^{32}P]GTP$, $K_m$ and $k_{cat}$ were determined by Lineweaver-Burk plots that in the temperature range 50–91°C were all linear. The data reported in Table I show that, at increasing temperatures both $k_{cat}$ and $K_m$ increased; the catalytic efficiency, expressed by the ratio $k_{cat}/K_m$, reached a maximum at 80°C. The possibility that the increase with temperature of $K_m$ of SsEF-2 GTPase for $[\gamma-^{32}P]GTP$ could also be due to a competitive inhibition by the GDP produced upon hydrolysis of $[\gamma-^{32}P]GTP$ was ruled out since such an amount was negligible compared to that required to get a significant increase of $K_m$ (see below). A Lineweaver-Burk plot gave a value for the energy of activation equal to 85 kJ/mol; at 87°C the calculated values for $\Delta H^*$, $\Delta S^*$, and $\Delta G^*$ were 82 kJ mol$^{-1}$, $-3$ J mol$^{-1}$ K$^{-1}$, and 83 kJ mol$^{-1}$, respectively. These energetic data were quite close to those reported for Escherichia coli EF-G GTPase at 30°C (19), thus indicating that the two enzymatic systems have similar energetic requirements.

It is remarkable that at the growth temperature of the respective source organisms, the affinity for GDP of GTPase is very similar between S. solfataricus SsEF-2 ($K_m = 32$ $\mu M$) and E. coli EF-G ($K_m = 41$ $\mu M$) (20).

Affinity of SsEF-2 for Guanosine Nucleotides in the Presence of Ribosomes. Evaluated by the Inhibition of GTPase by GDP and Gpp(NH)p—GTPase was competitively inhibited by both GDP and Gpp(NH)p, the latter being a stronger inhibitor (Fig. 4). At increasing concentrations of both inhibitors the value of $v$ remained unchanged, whereas $K_m$ for $[\gamma-^{32}P]GTP$, that was 9 $\mu M$ in the absence of inhibitors, increased to 18, 29, 47, and 93 $\mu M$ in the presence of 5, 10, 30, and 60 $\mu M$ GDP, and to 48 $\mu M$ and 312 $\mu M$ in the presence of 2 $\mu M$ and 32 $\mu M$ Gpp(NH)p. Values of 7 and 1 $\mu M$ were calculated for the inhibition constant, $K_i$, in the presence of GDP and Gpp(NH)p, respectively; they represent also the values of $K_i$, of SsEF-2-GDP and SsEF-2-Gpp(NH)p in the presence of ribosomes. In fact, the instability during filtration on nitrocellulose of the SsEF-2-GDP and SsEF-2-Gpp(NH)p complexes with ribosomes did not allow the directed determination of the respective $K_i$ values. The data reported in Table II show that ribosomes reverse the relative affinity of SsEF-2 for GDP and Gpp(NH)p; in fact, the affinity for GDP, that in the absence of ribosomes was 51-fold higher compared to that for Gpp(NH)p, in the presence of ribosomes became 7-fold lower. A similar behavior has been described for E. coli EF-G (3, 15) and rat liver EF-2 (16). The increased affinity of SsEF-2 for GDP might explain the stimulatory effect by the ribosome on the SsEF-2 GTPase; in addition, the decreased affinity for GDP should reduce the inhibitory effect by the GDP formed upon the hydrolysis of GTP.

Effect of ADP-ribosylation of SsEF-2 on the GTPase—Since the ADP-ribosylation of SsEF-2 inhibits poly(Phe) synthesis (7, 21), its effect on the SsEF-2 GTPase was investigated. The ADP-R-F2 was still able to sustain the GTPase whose kinetic was not greatly affected. In fact, at 60°C the values of $k_{cat}$ and $K_m$ for $[\gamma-^{32}P]GTP$ are 0.6 s$^{-1}$ and 12 $\mu M$, respectively (for a comparison with nonmodified SsEF-2, see Table I). ADP-R-F2 GTPase was competitively inhibited by GDP, and the value of $K_i$ was identical to that determined with not ADP-ribosylated SsEF-2. Therefore, the ADP-ribosylation of SsEF-2 does not affect the binding of GDP and GTP to SsEF-2; a similar finding has been reported for rat liver EF-2 (22).

To our knowledge, this is the first report on the characterization of an archaeal hyperthermophilic EF-2 regarding either its affinity for GDP and GTP or its ribosome-stimulated GTPase activity. Our recent report that the elongation factor SsEF-1a binds GDP and GTP (6) and elicits an intrinsic

### Table I

| Temperature (°C) | $k_{cat}$ (s$^{-1}$) | $K_m$ ($\mu M$) | $k_{cat}/K_m$ (s$^{-1}$ $\mu M^{-1}$) |
|-----------------|---------------------|----------------|-----------------------------------|
| 50              | 0.2                 | 1             | 0.2                               |
| 60              | 0.8                 | 8             | 0.08                              |
| 70              | 1.7                 | 10            | 0.17                              |
| 75              | 2.7                 | 11            | 0.25                              |
| 80              | 3.5                 | 13            | 0.27                              |
| 87              | 6.2                 | 32            | 0.19                              |
| 91              | 8.8                 | 148           | 0.06                              |

### Table II

| Source | Temperature (°C) | Ribosome | $K_a$ ($\mu M$) | Ref. |
|--------|-----------------|----------|----------------|------|
| S. solfataricus | 60              | –        | 1              | 51   | This work |
| S. solfataricus | 60              | +        | 7$^*$          | 43   | 15      |
| E. coli | 25              | –        | 36$^*$         | 4.3$^*$ | 3      |
| E. coli | 25              | +        | 1.6            | 2.8  | 24      | 16 |
| Rat liver | 23              | –        | 9.3            | 0.3  | 16      |
GTPase (13) and the data reported in the present work prove that the *S. solfataricus* elongation factors are functionally similar to the corresponding bacterial and eukaryal counterparts (1, 3, 5, 7) despite that the temperature for optimum growth of *S. solfataricus* is about 50 °C higher.

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