In vitro translation of halobacterial mRNA

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A system for in vitro translation of halobacterial mRNA is described. It is composed of 70S ribosomes, a nuclease-treated S100 supernatant, yeast tRNA and halobacterial mRNA. Specific expression of proteins could be demonstrated by translation of messages from halophage φ1-infected cells. However, under no conditions could a pure bacterio-opsin message be observed to be active in translation. This points to a more complex translational property of bacterio-opsin and possibly other membrane proteins in archaebacteria.

In vitro translation; Bacteriorhodopsion; Archaebacterium (Halobacterium halobium)

1. INTRODUCTION

Halobacterium halobium is an archaebacterium living in highly saline environments on the basis of fermentative, respiratory or phototrophic pathways. Photophosphorylation is mediated by the purple membrane system as a light-energy converter. The only protein in the purple membrane is bacteriorhodopsin containing a covalently bound retinal molecule (for review see [1,2]). As in all phototrophic bacteria the photosynthetic apparatus also in halobacteria is regulated by light and oxygen. Both retinal and opsin synthesis depend in a complicated mechanism on these two parameters. Dropping oxygen tension in the medium causes bacterio-opsin to be the main product of translation and the bacterio-opsin mRNA becomes the prominent transcript [3,4]. In order to dissect the regulatory phenomena involved in the biosynthesis of the purple membrane system, an in vitro translation system for halobacteria would be helpful. An endogenous mRNA-dependent cell-free translation system was first established by Bayley and Griffith [5] for *Halobacterium cutirubrum*. In this system externally added poly-U was added as a matrix-directed incorporation of [^{14}C]phenylalanine into precipitable material. Kessel and Klink [6] improved the system and used it as an assay for isolation of the halophilic elongation factors 1 and 2. Saruyama and Nierhaus [7] developed a highly active poly-U-dependent system but this in vitro system failed to translate exogenously added mRNA. Here we report on mRNA-dependent protein synthesis, initiation of translation and show that isolated bacterio-opsin mRNA is not translatable in vitro.

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Abbreviations: SDS, sodium dodecyl sulfate; TCA, trichloroacetic acid; PPO, 2,5-diphenyloxazole; BO, bacterio-opsin; BR, bacteriorhodopsin; bop, bacterio-opsin gene; hop, halorhodopsin gene; brp, bacteriorhodopsin-related protein gene

2. MATERIALS AND METHODS

Glass-fibre filters GF/C were obtained from Whatman [3,4-^{35}S]methionine (1440 Ci/mmol) from Amersham and micrococcus nuclease (9000 units/mg) from PL-Biochemicals. All other chemicals were from Merck and of the purest degree available.

2.1. *Halobacterial strains*

Halobacterium halobium strains ET 1001, JW5 [8] and R_1 [9] were used.

2.2. *Preparation of the S_{40} halobacterial cell extract*

Cells (ET 1001 or JW5) were grown in a complete medium at 40°C with limited oxygen supply [10] to the late logarithmic growth phase (OD_{660} 0.4–0.5, Zeiss photometer). They were then pelleted at 9000 × *g* for 10 min and washed according to the procedure described in [5] once with basal salt (4.2 M NaCl, 80 mM $MgSO_4 \cdot 7H_2O$, 20 mM KCl and 10 mM trisodium citrate, pH 7.0) and once with translation buffer (10 mM Hepes, pH 7.6, 3.4 M KCl and 100 mM magnesium acetate). The cells were resuspended in translation buffer by adding 1.5 vols/g wet weight and dialysed at 4°C for 4 h against 3 changes of translation buffer. All cells lysed within this time as checked microscopically. The suspension was centrifuged at 4°C for 1 h at 40000 × *g* and the supernatant stored in aliquots at −70°C (fraction S_{40}).

2.3. *Isolation of ribosomes from S_{40}*

The S_{40} supernatant (1 ml, 0.6 ml/g wet weight of cells, A_{260} = 38.8, A_{260}/A_{280} = 1.5) was centrifuged at 100000 × *g* for 16 h at 4°C. The supernatant (S_{100}, A_{260} = 16.85) was frozen in aliquots and stored at −70°C. The pellet was resuspended in 150 μl translation buffer and also stored at −70°C. Assuming that 1 A_{260} unit (1 ml solution of OD = 1 at 260 nm) of this suspension corresponds to 24 pmol 70 S ribosomes [7] 0.33 nmol ribosomes were isolated from 1 g cells (wet weight) and 1 ml of S_{40} contained 490 pmol 70 S ribosomes.

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2.4. *Micrococcal nuclease treatment of S_{100}*

S_{100} (40 μl, 0.06 A_{260} units) was incubated for 3 h at 37°C with 150 units micrococcal nuclease (150000 units/ml 50 mM glycine, pH 8.2, and 5 mM $CaCl_2$) in the presence of 22 mM calcium chloride. The reaction was stopped with 44 mM EGTA.

2.5. *Complete translation system*

Micrococcal nuclease-treated S_{100} (0.06 A_{260} units, 40 μl) was mixed with 6–20 pmol 70 S ribosomes (up to 6 μl) and 20 μg of yeast tRNA (2 μl).

2.6. *Incubation and assay conditions*

Either 40 μl of S_{40} (1.16–1.5 A_{260} units) or 40 μl of the complete translation system were mixed with 170 μl translation mix (1.4 M KCl, 28 mM magnesium acetate, 41 mM Hepes, pH 7.6, 141 mM ammonium sulfate, 1.17 M ammonium chloride, 29 μM amino acids without methionine, 0.05–2 μCi [^{35}S]methionine (1440 Ci/mmol), 7 mM sodium ATP, 1 mM sodium GTP, 7 mM sodium phosphoenol pyruvate and 30 mg of solid KCl) and incubated for 1 h at 37°C. For determination of incorporated [^{35}S]methionine 10 μl of the reaction mixture were added to 200 μl 50 mM NaOH and precipitated after 15 min with 1 ml ice-cold 25% TCA. The precipitates were collected on glass-fibre filters, washed twice with 5% ice-cold TCA, dried and counted in a scintillation counter. If samples were to be loaded on a polyacrylamide gel the incubation mixture was diluted with 200 μl water, precipitated with 25% ice-cold TCA and centrifuged at 9000 × *g* for 10 min. The pellet was washed twice with 5% ice-cold TCA, twice with ethanol, dried and dissolved after neutralization with NaOH in 30 μl 10% SDS. This procedure did not remove smaller molecular weight proteins from the precipitate as was shown with in vivo-labelled protein extracts as controls. The sample was heated for 5 min to 90°C with 30 μl sample buffer [11] and loaded on a 9–24% polyacrylamide SDS gel. After electrophoresis the gels were treated with PPO, dried and autoradiographed (Linhardt X-Ray 90).

2.7. *In vivo labelling of bacterio-opsin*

200 ml of an ET1001 or JW-5 cell suspension was grown in complete medium at 40°C with limited oxygen supply [10] to their late logarithmic growth phase (OD_{660} = 0.4–0.5) according to the procedure described in [3]. Cells were pelleted at 9000 × *g* for 10 min, washed once with basal salt and resuspended in 200 ml basal salt. The suspension was shaken for 4 h at 40°C to exhaust amino acid pools. The cells were concentrated to an OD_{660} of 2.0 in basal salt containing 0.05% alanine.

The suspension (5 ml) was incubated at 37°C with 5 μCi [^{35}S]methionine (1440 μCi/mmol) and slowly shaken before the reaction was stopped by addition of 3 ml ice-cold basal salt. After centrifugation at 9000 × *g* for 3 min at 0°C the pellet was lysed by H_2O (200 μl) in the presence of DNase. If the samples were loaded on an SDS polyacrylamide gel 5 μl of the lysate was mixed with 30 μl of sample buffer [10] and heated for 5 min at 95°C.

2.8. *Isolation of total RNA and partial purification of bacterio-opsin mRNA*

The RNA was isolated from cells of strain ET 1001 at the late logarithmic growth phase (OD_{660} = 0.5) according to [4]. Cells (6 g wet weight) were lysed in 60 ml of guanidium thiocyanate buffer (4 M guanidium thiocyanate/25 mM sodium citrate, pH 7.0/0.05% *N*-laurylsarcosine/0.3% antifoam A/0.7% mercaptoethanol) and centrifuged (130000 × *g* for 20 h) through a cushion of 5.7 M CsCl buffered with 25 mM sodium acetate, pH 5. The RNA pellet was dissolved in guanidium hydrochloride buffer (7.5 M guanidium hydrochloride, neutralized with NaOH and buffered with 25 mM sodium citrate, pH 7.0, and 5 mM dithiothreitol). The solution was adjusted to pH 5.0 by adding 0.025 vols of 1 M acetic acid and the RNA was precipitated with 0.5 vols of ethanol. After centrifugation for 10 min at 900 × *g* this process was repeated before the RNA was dissolved in sterile water and precipitated again with 0.1 M sodium acetate, pH 5.0, and 2 vols of ethanol.

For partial purification of bacterio-opsin mRNA 200 A_{260} units of total RNA were dissolved in 500 μl of 25 mM Hepes, pH 7.6, 0.1 M LiCl and 0.5% SDS, layered on a linear sucrose density gradient (7–30% in the same buffer) and centrifuged for 20 h at 113000 × *g* at 20°C. Fractions were collected from the top of the gradient by pumping a 50% sucrose solution from the bottom into the tube. The RNA was precipitated with 0.1 M sodium acetate, pH 5.0, and 2 vols ethanol and the fractions containing the bacterio-opsin mRNA identified by filter hybridization. The bacterio-opsin gene probe was provided by H. Vogelsang-Wenke and total RNA of R_1 and R_1 infected with the halobacterial phage ϕH_1 by F. Gropp. For this, 500 ml of cells were grown to late logarithmic phase (OD_{660} = 0.9) and infected with a moi of 10 for 5 h. Total RNA was then isolated as described above.

3. RESULTS

Based on early work of Bayley and Griffith [5] we isolated the S_{40} fraction from a crude cell lysate using bacterio-opsin overproducing halobacterial strains ET 1001 or its retinal-deficient derivative JW 5 by dialysing the cells against 3.4 M buffered KCl containing 0.1 M magnesium acetate followed by centrifugation at 40000 × *g* for 1 h. This procedure made DNase treatment and homogenization [5] unnecessary and yielded an S_{40} fraction containing 490 pmol 70 S ribosomes/ml. Fig.1 shows the incorporation of [^{35}S]methionine into TCA-precipitable material. The maximum of incorporation was reached after 45 min with an incorporation of about 6 fmol [^{35}S]methionine/pmol ribosome in this particular experiment.

The in vitro translation products were separated by SDS-PAGE and occurred as defined patterns of bands shown in fig.2A. The main products have relative molecular masses of 96000, 80000, 62000 and 56000. Surprisingly no bacterio-opsin is synthesized although the cell extract was prepared from cells exhibiting a high rate of bacterio-opsin production and therefore were expected to have high bacterio-opsin mRNA concentration (see fig.2B). A preparation of membrane bound polysomes also failed to demonstrate bacterio-

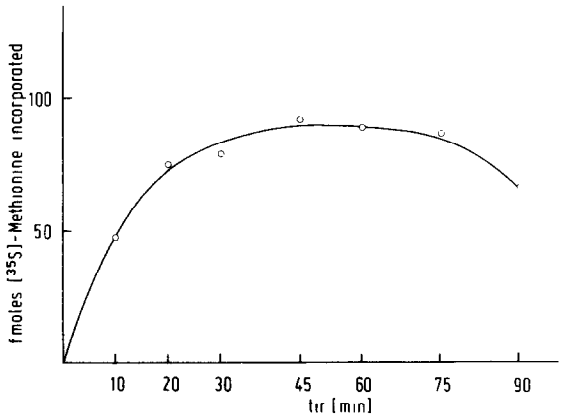

Fig.1. Time course of [^{35}S]methionine incorporation into protein. 40 μl of S_{40} were incubated at 37°C with 170 μl translationo mix and 2 μCi [^{35}S]methionine. Incorporation was determined as described in section 2.

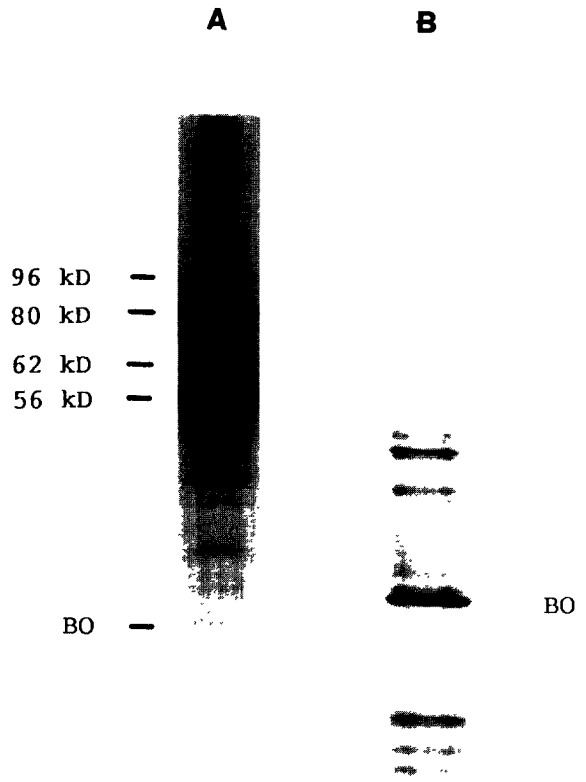

Fig.2. Autoradiography (polyacrylamide gel 9–24%). (A) In vitro translated proteins of ET 1001 S_{40}. (B) In vivo labelled proteins.

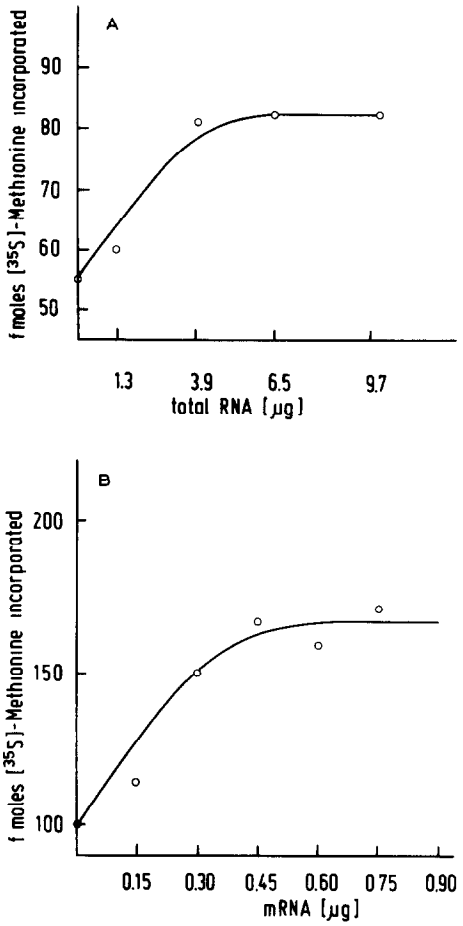

Fig.3. Total RNA (A) and mRNA-dependent (B) incorporation of [^{35}S]methionine into protein. Micrococcal nuclease-treated S_{100} was reconstituted with isolated ribosomes (20 pmol) and 20 mg yeast tRNA and incubated with variable amounts of mRNA and total RNA. (A) mRNA dependence. (B) Total RNA dependence.

opsin synthesis under our experimental conditions. In order to show that this block in bacterio-opsin mRNA translation is of physiological relevance a specific translation system was established.

Addition of mRNA to the S_{40} fraction did not enhance [^{35}S]methionine incorporation and did not produce specific translation products. Furthermore, preincubation of the mixture did not improve the situation. Therefore, the S_{40} fraction was treated with micrococcus nuclease to remove endogenous mRNA. Due to high salt conditions which inhibit non-halophilic enzyme activities a high dose of micrococcus nuclease (150 units) was added to the extract and incubation continued for 3 h at 37°C. This procedure rendered the supernatant dependent on exogenously added mRNA in protein synthesis but results were not always reproducible because ribosomes were occasionally inactivated by the nuclease treatment. Therefore 70 S ribosomes were isolated from the S_{40} fraction by centrifugation at 100000 × g for 16 h before subjecting the remaining S_{100} supernatant to micrococcus nuclease digestion. A complete translation system was then reconstituted by adding back the ribosomes together with yeast tRNA and halobacterial mRNA or total RNA.

Table 1

Incubation components	fmol [^{35}S]methionine
S_{40}	77
S_{100}	35
Ribosomes	19.9
S_{100}/ribosomes	65
S_{100}, micrococcal nuclease-treated	10.9
Complete system	24
Complete system/R_1-RNA	90
Complete system/R_1/ϕH_1-RNA	100

Table 1 compares the different fractions and complemented systems for their translational activity. As mentioned, the S_{40} fraction has considerable activity but does not show dependence from exogenously added mRNA. The activity of S_{100} is correspondingly decreased and ribosomes alone retain only about 20% relative activity. Combination of the S_{100} fraction with ribosomes increases the activity almost to the level of the S_{40} fraction, whereas treatment of the S_{100} fraction with micrococcal nuclease decreases activity to the

lowest level observed (11%). The complete system with ribosomes and micrococcal nuclease-treated S_{100} fraction exhibits 24% activity and a strong increase upon addition of mRNA from various sources. Fig.3 shows the saturation of this system with either total RNA (A) or mRNA (B). The translational activity by added RNA is increased by a factor of 3–4. The average activity of the reconstituted translation system was 12 fmol [^{35}S]methionine incorporated/pmol ribosome. At an average methionine content of proteins of 2.2% a value of 600 fmol amino acids incorporated/pmol ribosome is obtained. This value is about 10–20-fold lower than the poly-U-dependent phenylalanine incorporation reported by Saruyama and Nierhaus [7].

The in vitro translation products obtained in the complete RNA-complemented system are shown in fig.4. Lane A shows the products derived from remnant endogenous RNA, lane B proteins made upon addition of total RNA isolated from strain R_1. Comparison of lanes A and B shows that not only existing bands in A are enhanced in B but, in addition, new bands appear (marked by arrows). Lane C shows proteins translated from RNA isolated from R_1 cells after phage infection. One additional protein at 80000 is synthesized (marked by an asterisk) which may be a phage-specific product. The main coat protein of phage ϕH has a molecular mass of about 80000 [12]. All attempts to show specific translation for bacterio-opsin mRNA after partial purification on a sucrose density gradient were not successful although the experiment shown in fig.4 demonstrated that initiation of translation is possible under these conditions. This corroborates the fact that already mRNA isolated from cells during a phase where the opsin messenger must be the most prominent one (fig.2A) does not allow translation of the opsin polypeptide chain although the control experiment shows that the ϕH-coded message apparently can be translated (fig.4, lane C).

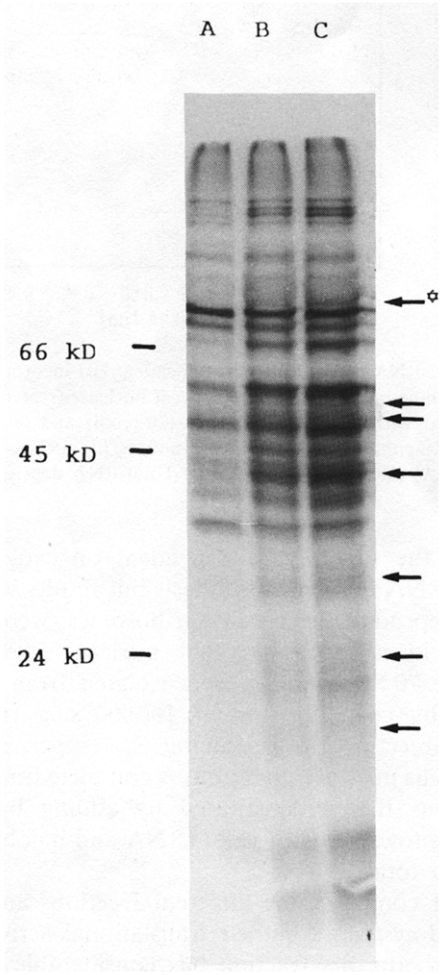

Fig.4. Autoradiography of in vitro translated proteins. Components were incubated for 1 h at 37°C with 170 μl translationo mix and 0.05 μCi [^{35}S]methionine. (A) S_{100}, treated with micrococcal nuclease and reconstituted with 7.4 pmol ribosomes and 20 mg yeast tRNA; (B) same samples as in (A) but incubated with total RNA (4 μg) isolated from strain R_1; (C) same sample as in (A) but incubated with total RNA (4 μg) isolated from the strain R_1 after 5 h infection with the phage ϕH_1.

4. DISCUSSION

So far only two halophilic membrane proteins were characterized: bacterio-opsin (bop) and halo-opsin (hop) [13]. On the gene level a third putative membrane protein, bacterio-rhodopsin-related protein (brp), which is involved in regulation of bop transcription has been investigated [14,15]. The mRNAs of all 3 genes share unusual properties which differ e.g. from that of the mRNA endocing the large subunits for the DNA-dependent RNA polymerase [16]. The transcripts in the case of bop, hop and brp start a few nucleotides upstream from the start codon AUG without a ribosomal binding site in the non-translated region. Furthermore, the 5′-ends can form hairpin loop structures to an extent of 14 base-pairing nucleotides. Whether these secondary structures have physiological importance is not clear, but at least they provide a possible explanation for the non-translatability of the bop mRNA. In this case one has to assume that during preparation of the total RNA fraction a factor is lost or inactivated which is responsible to present the RNA to the ribosome in a translatable form. Such a factor is characteristic for translation in processing of eukaryotic membrane proteins. Their translation is arrested by a signal recognition particle (SRP) until the entire complex is bound to the endoplasmic reticulum [17]. It is worth mentioning that *Halobacterium halobium* contains a prominent 7 S transcript of unknown function with striking homologies to the 7 S RNA which is part of the eukaryotic signal recognition particle [18]. But neither the bop nor the hop transcripts have typical eukaryotic signal sequences and it is even doubtful whether the first 15 amino acids

serve as a signal sequence at all. Since the hop gene and therefore its message is available it would be interesting to demonstrate whether this block in translation is specific for the bop mRNA or is also found for the hop message and, even more general, is typical for all halophilic membrane proteins. To this end, factors must be considered which might be necessary for the proper folding state of bacterio-rhodopsin and other membrane proteins prior to insertion into the cell membrane. As an alternative translational control could be limited to the members of the photosynthetic and phototactic apparatus of the halobacterial cells regulated by the oxygen level of the medium.

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