Vol. 99, No. 2, 1981 BIOCHEMICAL AND BIOPHYSICAL RESEARCH COMMUNICATIONS
March 31, 1981 Pages 722-728

THE EFFECT OF ELEVATED TEMPERATURE ON PROTEIN SYNTHESIS IN CELL-FREE EXTRACTS OF CULTURED CHINESE HAMSTER OVARY CELLS.

James S. Hutchison and Kivie Moldave

Department of Biological Chemistry, University of California, Irvine College of Medicine, Irvine, California 92717

Received February 18,1981

SUMMARY: The effect of elevated temperature on the activity of various components involved in protein synthesis was investigated in extracts from cultured Chinese hamster ovary cells. The translation of exogenous mRNA was markedly inhibited by preincubation of the extract for 15 to 20 minutes at 42°C. However, the following intermediary reactions were not affected, or only slightly inhibited, at 42°C: 1) the incorporation of Met-tRNA$_f$ into eIF-2·Met-tRNA$_f$·GTP ternary complex; 2) the interaction of the ternary complex with 40S ribosomal subunits to form the 40S preinitiation intermediate; 3) the binding of mRNA and 60S subunits to form the 80S initiation complex; and 4) the reactions catalyzed by elongation factors EF-1 and EF-2. The activity of Met-tRNA synthetase was markedly inhibited, affecting the formation of initiator Met-tRNA$_f$ required for the initiation of protein synthesis and the translation of natural mRNA. Other aminoacyl-tRNA synthetases were not significantly affected by the elevated temperature.

INTRODUCTION

Numerous reports have described the effects on protein synthesis when mammalian cells were incubated at elevated temperatures (1-9). Most of these studies indicated that peptide chain initiation was inhibited at temperatures near or above 40°C, although in some experiments polysome disaggregation was not observed (1,6), and in others, evidence for an effect on chain elongation was also obtained (8). Many of these interpretations were based on indirect analyses which did not involve direct measurements of intermediary reactions or components involved in protein synthesis, and the direct effect of high temperature on translational components in cell-free systems was not investigated. The development of a cell-free system from cultured mammalian cells that actively and accurately translates exogenous natural mRNAs and synthetic polynucleotide templates (10), and the development of procedures for the analysis of intermediary reactions involved in initiation, elongation and termination of protein synthesis (10,11) allow a detailed analysis of the manner in which elevated temperatures affect various translational components in normal cells. Such information would be of particular interest in view of the increasing availability of temperature-sensitive mutants for studies of protein synthesis (12-15). This report describes the analysis of transla-

0006-291X/81/060722-07$01.00/0
Copyright © 1981 by Academic Press, Inc.
All rights of reproduction in any form reserved.

tional reactions in cell-free preparations from Chinese hamster ovary cells, and the effects of elevated temperature on this process.

MATERIALS AND METHODS

The growth of Chinese hamster ovary (CHO, GAT$^-$) cells, preparation of the postmitochondrial (S-30) extract, degradation of endogenous mRNAs with micrococcal nuclease, and incubation conditions and procedures for the translation of various exogenous templates, have been described in detail (10). Translation of natural (globin) mRNA was assayed in incubations containing nuclease-treated extract (60-100 μg protein), buffered-salts, dithiothreitol (1 mM), ATP (0.9 mM), GTP (0.18 mM), creatine phosphate (18 mM), creatine phosphokinase (24 μg), 19 non-isotopic amino acids (0.1 mM, excluding leucine), 1.4 μM [^{3}H]leucine (51.6 Ci/mmole) and reticulocyte poly(A)$^+$ mRNA (0.2-0.4 μg). Translation of poly(U) was assayed in similar incubations except that the Mg^{++} concentration was raised to 8.5 mM, the radioactive amino acid was [^{3}H]phenylalanine (5 μM; 15 Ci/mmole) and the template was polyuridylic acid (25 μg). The incubation volumes were 0.05 ml. At the end of the incubation at 30°C for 60 minutes, the hot (95°C) 5% trichloroacetic acid-insoluble protein fraction was prepared and counted (16). The addition of globin mRNA or poly(U) to nuclease-treated extracts stimulated amino acid incorporation over 40- to 50-fold.

In some experiments, analyzed by gradient centrifugation, the equivalent of 5 incubations (0.25 ml) were combined. The mRNA-depleted postmitochondrial extract was incubated with 0.3 μM [^{35}S]methionine (1380 Ci/mmole) or 19 μg of [^{35}S]Met-tRNA$_f$ (33,500 cpm/μg of tRNA) in the absence of mRNA, or with globin mRNA plus 1.5 mM cycloheximide. The incubation mixtures were then fixed with 0.2% formaldehyde, layered on and centrifuged through linear sucrose gradients, individual gradient fractions were filtered through Millipore membranes, and counted (10,11). Cycloheximide did not appear to affect the formation of intermediary complexes in chain initiation.

Aminoacyl-tRNA synthetase activities in the high-salt cytosol extract (0.5 M KCl, 130,000g supernatant; 30 min., Beckman Airfuge), were assayed by incubating with uncharged rat liver tRNA (6.5 μg), with and without ATP (0.9 mM) and 0.14 μM [^{35}S]methionine (1120 Ci/mmole), [^{3}H]leucine (3.9 μM, 51.6 Ci/mmole), or [^{3}H]phenylalanine (13 μM, 15 Ci/mmole). After 60 minutes at 30°C, the cold (2°C) acid-insoluble fractions were isolated and counted. Values presented were corrected by subtracting the amount of radioactivity obtained in the absence of ATP, usually less than 5% of that obtained with ATP.

RESULTS AND DISCUSSION

The effect of preincubation temperature on the subsequent ability of cell extracts to translate mRNA at 30°C is summarized in Table I. Extracts preincubated at 30°C had 85-90% of the globin mRNA-translating activity in extracts maintained at 2°C; after preincubation at 42°C, however, only about 20% of the activity remained. Preincubation at 30°C or 42°C had no significant effect on the translation of poly(U). These results suggested that the activities required for chain elongation such as elongation factors EF-1 and EF-2, ribosomes, and phenylalanyl-tRNA synthetase were not as sensitive to the elevated temperature as a component or components involved in chain initiation or in the formation of initiator Met-tRNA$_f$.

Table I. The effect of preincubation of postmitochondrial extract at elevated temperature, on the translation of exogenous natural and synthetic polynucleotide templates.

Preincubation temperature	pMol of amino acid incorporated into protein	
	[^{3}H]leucine + globin mRNA	[^{3}H]phenylalanine + poly(U)
2°	3.18	27.57
30°	2.73	28.62
42°	0.65	24.82

The S-30 fractions were maintained for 20 minutes at the temperature noted, then treated with nuclease to deplete endogenous mRNAs and incubated with [^{3}H]leucine plus globin mRNA or [^{3}H]phenylalanine plus poly(U) as described in the text.

The reactions required for initiation of protein synthesis were assayed by examining the formation of intermediary (40S preinitiation and 80S initiation) complexes (10,11) with radioactive methionine, as shown in Figure 1. In the absence of mRNA, the amount of [^{35}S]methionine-containing material recovered in the 5-6S region (fractions 1-7), representing primarily eIF-2·Met-tRNA$_f$·GTP ternary complex (and possibly some synthetase-bound Met-tRNAs) was about 60% lower in the 42°C-preincubated (panel C) than in the control (panel A) extract; the intermediate representing the complex containing the ternary

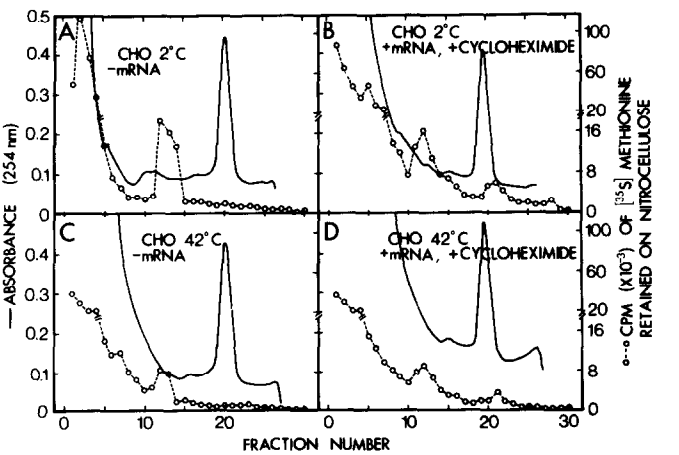

Figure 1. The effect of preincubation of postmitochondrial extract at elevated temperature, on the formation of initiation intermediates in protein synthesis. The S-30 fractions were maintained for 10 minutes at 2°C (A and B) or 42°C (C and D), then treated with nuclease and incubated with [^{35}S]-methionine in the absence of mRNA (A and C) or with globin mRNA plus cycloheximide (B and D). Gradient centrifugation, spectrophotometric analysis (solid lines), and determination of radioactive complexes by Millipore filtration (circles, broken lines), as described in the text.

complex bound to 40S subunits (fractions 11-15) was also about 50% lower in the heated extract (C) than in samples maintained at 2°C (A). In the absence of mRNA, radioactive methionine was not transferred to 80S ribosomes. Incubations in the presence of globin mRNA and cycloheximide (to prevent protein synthesis) revealed three peaks containing radioactive methionine (panels B and D); in addition to the 5-6S and the 40S material, a peak representing the 80S initiation complex (fractions 19-23) was also recovered. The amounts of radioactive methionine in each of the three complexes were 40-60% lower in the 42°C-preincubated samples (D) than in the control extract maintained at 2°C (B). The amount of [^{35}S]methionine recovered in the 40S and 80S ribonucleoprotein particles, as percent of the total [^{35}S]methionine-containing complexes in the gradient, was very similar in both the heated and control extracts. For example, 20 and 23%, respectively, of the total radioactive methionine recovered was obtained in the 40S plus 80S peaks in both the heated (42°C) and control (2°C) extracts. The amounts bound to the 80S complex, as percent of the total ribonucleoprotein particle-bound [^{35}S]-methionine (40S plus 80S), were also very similar; 25 and 26%, respectively, of the total particle-bound methionine was associated with the 80S initiation complex in both extracts.

The results obtained from the gradient centrifugation analysis above indicated that the formation of the eIF-2·Met-tRNA$_f$·GTP ternary complex with radioactive methionine was markedly decreased by preincubation of the extract at 42°C; this finding suggested either that eIF-2 was inactivated at the elevated temperature or that Met-tRNA synthetase was inactivated and therefore unable to form the Met-tRNA$_f$ precursor necessary for ternary complex formation. To bypass the requirement for the Met-tRNA synthetase, experiments were carried out with preformed, partially purified [^{35}S]Met-tRNA$_f$ instead of free methionine. The effects of temperature and time of preincubation on the ability of postmitochondrial extracts to form complexes (eIF-2·GTP·Met-tRNA$_f$ and 40S preinitiation complex) with initiator Met-tRNA$_f$ are presented in Figure 2. In contrast to the experiment with [^{35}S]methionine (Figure 1) which showed a 60% decrease in ternary complex formation as a result of 42°C preincubation, the complex with Met-tRNA$_f$ recovered in the 5-6S region in this experiment was only about 20-30% lower, and that in the 40S region was about 25% lower in the 42°C-preincubated extract (B) than in the 2°C control (A). The percent of total [^{3}H]Met-tRNA$_f$ that was bound to 40S subunits in the two samples was quite similar; in the extracts maintained at 2°C (A), 32% of the Met-tRNA$_f$ recovered from the gradient was associated with 40S subunits, while in the extract preincubated at 42°C (B) 34% of the Met-tRNA$_f$ was bound

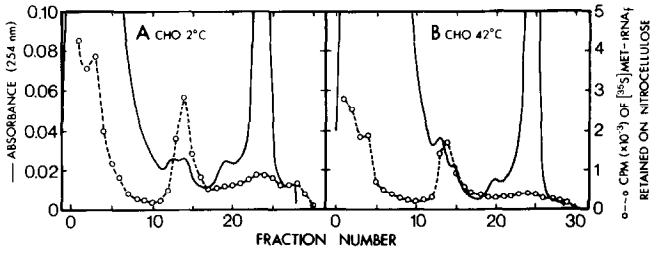

Figure 2. The effect of preincubation at elevated temperature, on the binding of [^{3}H]Met-tRNA$_f$ to preinitiation intermediates. The S-30 fractions were maintained for 20 minutes at 2°C (A) or 42°C (B), then treated with nuclease and incubated with [^{3}H]Met-tRNA$_f$, in the absence of mRNA, as described in the text. At the end of the incubation, reactions were analyzed by gradient centrifugation and Millipore filtration.

to 40S subunits. The recovery of Met-tRNA$_f$ in the 5-6S region, primarily ternary complex, which did not require the participation of Met-tRNA synthetase, was somewhat decreased by preincubation at 42°C, but not as much as when free methionine was the substrate. The small decrease in the amount of Met-tRNA$_f$ in the 5-6S region could be accounted for, to some extent, by the formation of a complex between Met-tRNA synthetase and Met-tRNA; thus, inacti-

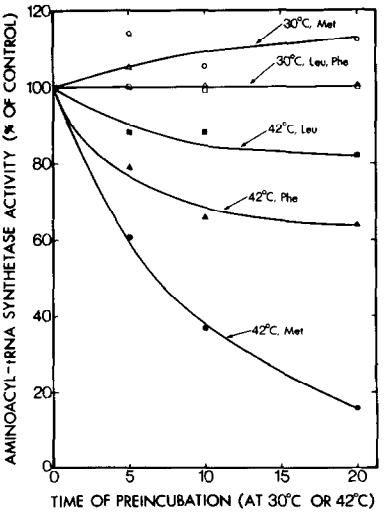

Figure 3. The effect of time and temperature of preincubation of cytosol, on the aminoacylation of various tRNAs. The high salt 130,000g extract was preincubated for varying periods of time at 30°C (open symbols) or 42°C (closed symbols), then for 60 minutes with a supplement of tRNAs (rat liver), and radioactive methionine (circles), leucine (squares), or phenylalanine (triangles), in the presence and absence of ATP. Analyses for aminoacyl-tRNAs were carried out as described in the text.

vation of the enzyme could be reflected by a decrease in radioactivity in this gradient peak. The subsequent binding of ternary complex to 40S subunits, however, was dependent only on the amount of ternary complex formed, and was not affected by preincubation at 42°C.

The above data suggested that reactions and components required for chain elongation and for the formation of initiation intermediates from Met-tRNA$_f$ did not seem to be responsible for the marked loss of translational activity observed at 42°C; the inactivation of Met-tRNA synthetase, however, remained as a likely possibility. Therefore, the effects of time and temperature of preincubation on the activity of Met-tRNA synthetase and several other aminoacyl-tRNA synthetases were examined (Figure 3). Preincubation at 30°C did not appear to affect significantly the aminoacylation reaction with methionine, phenylalanine, or leucine. In cytosol fractions preincubated at 42°C, leucine activation was decreased about 20% in 20 minutes, and that of phenylalanine was decreased about 40%. However, Met-tRNA synthetase was inactivated very rapidly; 50% of the activity was lost in about 7 minutes, and less than 20% of the activity remained after 20 minutes at 42°C.

A review of all of the reactions in protein synthesis that were investigated, and the manner in which they responded to the elevated temperature, indicated three distinct types of behavior at 42°C. The activity of ribosomes, Phe-tRNA synthetase, and elongation factors EF-1 and EF-2, as well as the binding of eIF-2·Met-tRNA$_f$·GTP ternary complex to 40S ribosomal subunits, and the subsequent reactions with mRNA and 60S subunits to form the 80S initiation complex, were not affected (less than 20%) by preincubation at 42°C. The formation of ternary complex from Met-tRNA$_f$, eIF-2 and GTP, and the aminoacylation of tRNA with leucine and phenylalanine were slightly inhibited (between 30 and 40%) by the elevated temperature. However, the translation of exogenous natural mRNA and the aminoacylation of tRNA with methionine were markedly (70-80%) inhibited by preincubation at 42°C for less than 20 minutes. These observations suggest that the inactivation of Met-tRNA synthetase is responsible for the temperature-dependent loss of ability to translate natural mRNA, due to the failure to generate Met-tRNA$_f$ for the process of chain initiation.

ACKNOWLEDGMENTS

The technical assistance of Eva Mack and Tom Ho is gratefully acknowledged. This work was supported in part by Research Grants AM 15156 and AG 00538 and Training Grant T32 CA 09054 (J.S.H.) from the National Institutes of Health.

REFERENCES

1. Canconi, F.M., Bank, A. and Marks, P.A. (1966) J. Mol. Biol. 17, 525-540.
2. McCormick, W. and Penman, S. (1969) J. Mol. Biol. 39, 315-333.
3. Schochetman, G. and Perry, R.P. (1972) J. Mol. Biol. 63, 577-590.
4. Sprechman, L.M. and Ingram, V.M. (1972) J. Biol. Chem. 247, 5004-5009.
5. Craig, N. (1975) Cell 4, 329-335.
6. Craig, N. (1976) J. Cell Physiol. 87, 157-166.
7. Craig, N. and Fahrman, C. (1977) Biochim. Biophys. Acta 474, 478-490.
8. Oleinick, N. (1979) J. Cell Physiol. 98, 185-192.
9. Henle, K.J. and Leeper, D.B. (1979) Cancer Res. 39, 2665-2674.
10. Fischer, I., Arfin, S.M. and Moldave, K. (1980) Biochemistry 19, 1417-1425.
11. Gasior, E., Herrera, F., McLaughlin, C. and Moldave, K. (1979) J. Biol. Chem. 254, 3970-3976.
12. Thompson, L.H., Harkins, J.L. and Stanners, C.P. (1973) Proc. Natl. Acad. Sci. U.S.A. 70, 3093-3098.
13. Haralson, M.A. and Roufa, D.J. (1975) J. Biol. Chem. 250, 8618-1823.
14. Thompson, L.H., Lofgren, D.J., and Adair, G.M. (1977) Cell 11, 157-168.
15. Ashman, C.R. (1978) Somatic Cell Genetics 4, 299-312.
16. Sadnik, I., Herrera, F., McCuiston, J., Thompson, H.A. and Moldave, K. (1975) Biochemistry 14, 5328-5335.