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PROTEIN SYNTHESIS IN RABBIT RETICULOCYTES XXII†: A HEAT STABLE DIALYZABLE FACTOR (EIF-1*) MODULATES Met-tRNA$_f$ BINDING TO EIF-1

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Summary

The peptide chain initiation factor EIF-1 forms a ternary complex, Met-tRNA$_f$•EIF-1•GTP in the absence of Mg^{++} and the preformed complex is stable to Mg^{++}. However, with homogeneous preparations of EIF-1, addition of Mg^{++} during the initial formation of the ternary complex strongly inhibits the complex formation.

A heat stable dialyzable factor (EIF-1*) which mostly remains associated with the high molecular weight protein complex, EIF-2 (TDF) during purification of the peptide chain initiation factors, has been purified using a phenol extraction procedure. EIF-1* restores the Met-tRNA$_f$ binding activity of EIF-1 in the presence of 1 mM Mg^{++}; in the presence of EIF-1*, Met-tRNA$_f$ binding by EIF-1 shows a sharp Mg^{++} optimum around 1 mM. EIF-1* is heat stable, alkali stable, dialyzable and pronase sensitive. The same EIF-1* preparation also strongly inhibits Met-tRNA$_f$ binding to EIF-1 in the absence of Mg^{++} and stimulates protein synthesis in a mRNA-dependent rabbit reticulocyte lysate system.

The first step in peptide chain initiation in eukaryotic cells is the formation of a ternary complex between a specific peptide chain initiation factor, EIF-1, Met-tRNA$_f$ and GTP (for a review see Ref. 4). The peptide chain initiation factor EIF-1 has been purified to homogeneity in several laboratories (5-7). The homogeneous EIF-1 preparation has a molecular weight of approximately 150,000 and is composed of three subunits of approximate molecular weights; 54,000, 52,000 and 38,000 (5-7). We have previously provided evidence that the ternary complex formation by EIF-1 is controlled

†Paper XXI in this series is Ref. 1

The nomenclature for the peptide chain initiation factors is according to Majumdar et al. (2). EIF-1, Met-tRNA$_f$ binding factor, also called eIF-2 according to Anderson et al. nomenclature (3); EIF-2 (TDF), ternary complex (Met-tRNA$_f$•EIF-1•GTP) dissociation factor; Co-EIF-1, stimulates Met-tRNA$_f$ binding to EIF-1.

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by at least two other protein factors: (1) Co-EIF-1. Stimulates Met-$tRNA_f$ binding to EIF-1 presumably by forming a stable quarternary complex, Met-$tRNA_f$·EIF-1·Co-EIF-1·GTP. In the presence of excess Co-EIF-1, one mol of EIF-1 binds one mol of Met-$tRNA_f$ (1,8). (2) EIF-2 (TDF). A high molecular weight protein complex catalyzes the dissociation of Met-$tRNA_f$·EIF-1 (Co-EIF-1)·GTP complex in the presence of high Mg^{++} (5 mM) and low temperature (0°C) (2) and is also required along with EIF-1 and Co-EIF-1 for AUG directed Met-$tRNA_f$ binding to 40S ribosomes (1,2).

The precise requirement of Mg^{++} in the formation of the ternary complex is not clear. With homogeneous preparations of EIF-1, the ternary complex formation is optimum in the absence of Mg^{++}. The complex thus formed is stable to Mg^{++} and dissociates in the presence of both EIF-2 and Mg^{++} (2). There are reports that ternary complex formation with EIF-1 may require Mg^{++} and several laboratories routinely use Mg^{++} during complex formation (6, 9, 10). However, ternary complex formation by our homogeneous preparations of EIF-1 is drastically inhibited by low concentration of Mg^{++}.

We have now isolated a low molecular weight protein factor, EIF-1*, from the high molecular weight protein complex, EIF-2 (TDF). This factor relieves the Mg^{++} inhibition of ternary complex formation. In the presence of EIF-1*, ternary complex formation by EIF-1 shows a sharp Mg^{++} optimum around 1 mM. EIF-1* is heat stable, alkali stable, dialyzable and pronase sensitive. The same EIF-1* preparation also stimulates protein synthesis in a mRNA-dependent reticulocyte lysate system.

Materials and Methods

The preparations of reticulocyte ribosomes and ribosomal 0.5 M KCl wash were the same as before (11). The peptide chain initiation factor, EIF-1, was purified to homogeneity following the procedure described previously (7,12). The homogeneous EIF-1 preparation showed three protein bands upon SDS-polyacrylamide gel electrophoresis corresponding to approximate molecular weights of 54,000, 52,000 and 38,000 (7). The peptide chain initiation factor EIF-2 (TDF) was purified using glycerol density (14-30%) gradient centrifugation followed by DEAE-cellulose chromatography as described previously (2). The preparation of Co-EIF-1 was also the same as described previously (1,8). The final preparation showed a single band upon SDS-polyacrylamide gel electrophoresis corresponding to an approximate molecular weight of 20,000 (1).

Purification of EIF-1*

During purification of the peptide chain initiation factors, most of the EIF-1* activity remains associated with the peptide chain initiation factor

EIF-2 (TDF) and sediments with the 450,000 molecular weight EIF-2 protein complex during the glycerol density gradient purification. The EIF-2 protein complex obtained by the glycerol density gradient procedure was concentrated further by passage through a small DEAE-cellulose column and one step elution with 0.3 M KCl in Buffer A. During this concentration, it was observed that the earlier fractions contained TDF (EIF-2) activity but the fractions immediately following TDF peak strongly inhibited Met-$tRNA_f$ binding to added EIF-1 when assayed by the standard assay method for EIF-2 in the absence of Mg^{++} in Stage I (2,7). The fractions showing the inhibitory activity were pooled and the solution was mixed with an equal volume of phenol (80%). The mixture was vortexed for 10 minutes and then centrifuged. The aqueous layer was saved. An equal volume of Buffer A (20 mM Tris-HCl, pH 8.0; 100 mM KCl, 50 μM EDTA, 5 mM β-mercaptoethanol and 5 percent glycerol) was then added to this phenol layer and the extraction procedure was repeated. The two aqueous fractions were pooled and an equal volume of ether was added to the solution to remove excess phenol. Ether extraction was repeated four times. Excess ether was removed by aeration. The solution containing EIF-1* activity was then concentrated by passage through a small DEAE-cellulose column equilibrated with Buffer A minus KCl followed by one step elution with Buffer A containing 0.3 M KCl.

Other materials and methods were the same as described previously (1,2,8,11).

Results

The peptide chain initiation factor EIF-1 forms a stable ternary complex, Met-$tRNA_f$·EIF-1·GTP, in the absence of Mg^{++}. However, with homogeneous preparations of EIF-1, we have recently observed that ternary complex formation by EIF-1 is strongly inhibited if Mg^{++} is present during the initial formation of the complex and addition of EIF-2 partially relieves this inhibition. This observation led us to the isolation of the factor EIF-1* from EIF-2 protein complex and to study its role in the regulation of the ternary complex formation. A typical set of data showing the roles of EIF-1*, and Co-EIF-1 on ternary complex formation at different Mg^{++} concentrations is shown in Fig. 1. Addition of EIF-1* strongly inhibited the ternary complex formation in the absence of Mg^{++} but almost completely relieved the inhibition of the complex formation in the presence of 1 mM Mg^{++}. The significance of the inhibition of complex formation in the absence of Mg^{++} and its stimulation at 1 mM Mg^{++} by EIF-1* is not apparent at present, and it is not clear how these two activities are related. However, in the presence of EIF-1*, the ternary complex formation by EIF-1 shows a sharp Mg^{++} optimum around

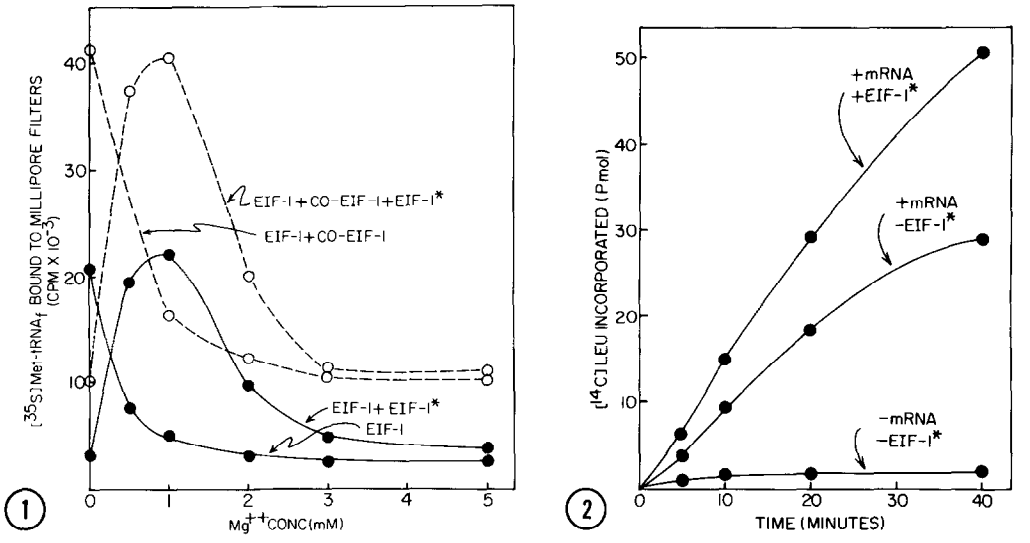

Fig. I. [^{35}S] Met-tRNA$_f$ binding to EIF-1 in the presence of EIF-1* and Co-EIF-1 at varying Mg^{++} concentrations. The standard Millipore filtration assay for [^{35}S] Met-tRNA$_f$ binding to EIF-1 was used (2,8,11). The incubation mixture contained, in a total volume of 0.072 ml:20 mM Tris-HCl, pH 8.0; 100 mM KCl; 10 µg bovine serum albumin; 2 mM dithiothreitol; 0.2 mM GTP; 6 pmol [^{35}S] Met-tRNA$_f$ (10,000 cpm/pmol); and where indicated EIF-1, 1.7 µg; Co-EIF-1, 2 µg and EIF-1*, 0.75 µg. The reaction mixture was incubated at 37° for 5 minutes at which time the reaction was terminated by addition of 3 ml of cold wash buffer (20 mM Tris-HCl, pH 8.0; 100 mM KCl). The solution was then filtered through a Millipore filter. The filter was washed twice with 10 ml of cold wash buffer, dried and counted for radioactivity.

Fig. II. Effect of EIF-1* on protein synthesis in a mRNA dependent rabbit reticulocyte lysate system. The mRNA dependent lysate was prepared according to the method of Pelham and Jackson (13). To 1 ml of freshly prepared lysate were added hemin, 25 µM, creatine phosphokinase, 50 µg/ml, calcium chloride, 1 mM and micrococcal nuclease 15 µg/ml. The lysate was then incubated for 15 minutes at 20°C and was then mixed with 2 mM EGTA (final concentration). 0.2 ml aliquots of the nuclease treated lysate were stored in liquid nitrogen. Rabbit hemoglobin mRNA was prepared by the method of Krystosek et al. (25). The final reaction mixture contained, in a total volume of 0.025 ml the following: 20 µM hemin, 0.2 mM GTP, 1 mM ATP, 10 mM creatine phosphate, 50 µg/ml creatine phosphokinase (213 I.U./mg), 100 mM KCl, 2 mM $MgCl_2$, 15 mM Tris-HCl (pH 7.6), 19 unlabelled amino acids and [^{14}C] Leu (specific activity, 440 cpm/pmol) in concentrations relative to their occurrence in rabbit hemoglobin, and where indicated 5 µg hemoglobin mRNA and 0.3 µg EIF-1*. The reaction mixture was incubated at 30° and 5 µl aliquots were assayed at different intervals by hot trichloroacetic acid precipitable radioactivity.

1 mM. This apparent stimulation of ternary complex formation by EIF-1* is not due to Co-EIF-1 activity. The addition of the protein factor, Co-EIF-1

TABLE I

Regulation of Met-tRNA$_f$ Binding to EIF-1 by

EIF-1*, Co-EIF-1 and EIF-2 (TDF)

Additional Factor(s)	Mg^{++} at Stage I (mM)	[^{35}S] Met-tRNA$_f$ Bound to Millipore Filters (pmol)	
		Stage I	Stage II
None	-	2.3	2.6
EIF-1*	-	0.5	0.6
Co-EIF-1	-	4.2	3.6
EIF-2	-	2.0	0.7
EIF-1* + Co-EIF-1	-	1.1	1.0
EIF-1* + EIF-2	-	0.4	0.3
Co-EIF-1 + EIF-2	-	3.9	1.2
EIF-1* + Co-EIF-1 + EIF-2	-	1.2	0.5
None	1	0.8	0.8
EIF-1*	1	2.0	2.0
Co-EIF-1	1	1.9	1.9
EIF-2	1	1.1	0.6
EIF-1* + Co-EIF-1	1	4.0	3.9
EIF-1* + EIF-2	1	1.8	0.8
Co-EIF-1 + EIF-2	1	3.1	1.1
EIF-1* + Co-EIF-1 + EIF-2	1	3.7	0.9

Standard two stage Millipore filtration assay method for Met-tRNA$_f$ binding to EIF-1 were used (2). Stage I incubation was at 37° for 5 minutes and where indicated 1 mM Mg^{++} was added to the reaction mixture. Stage II incubation was at the ice bath temperature and contained 5 mM Mg^{++}. Protein concentrations of the factors used were; EIF-1, 1.7 µg; EIF-1*, 0.75 µg; Co-EIF-1, 2 µg; EIF-2, 2.5 µg.

produced a general stimulation of EIF-1 activity under all the conditions tested. In the absence of Mg^{++}, the addition of Co-EIF-1 produced approximately two fold stimulation of EIF-1 activity whereas the addition of EIF-1* strongly inhibited ternary complex formation under the same conditions. The addition of Co-EIF-1 to EIF-1 did not protect the ternary complex formation from the inhibitory effect of Mg^{++} although the stimulatory effect of Co-EIF-1 on EIF-1 activity was observed at each Mg^{++} concentration and also in the presence of EIF-1*. It is clear from the data in Fig. 1 that while Co-EIF-1 produces a general stimulation of EIF-1 activity under all the conditions tested, addition of EIF-1* restores the EIF-1 activity of the inhibited system in the presence of 1 mM Mg^{++} to that observed at 0 mM Mg^{++}.

The results presented in Table I describe the roles of EIF-1*, Co-EIF-1 and also EIF-2 in the overall regulation of the ternary complex formation in the absence and presence of 1 mM Mg^{++}. As noted previously (2,7), EIF-2 (TDF) is a high molecular weight protein complex which catalyzes the dissociation of the ternary complex in the presence of high Mg^{++} concentrations (5 mM) and low temperature. The EIF-2 preparations used in these experiments were obtained after concentration of the glycerol gradient fractions by DEAE-cellulose chromatography described under Materials and Methods and only fractions which contained very little EIF-1* activity were used. It is clear from the results that EIF-2 (TDF) has no significant effect on the ternary complexes formed in the presence of 0 and 1 mM Mg^{++} and in different combinations with Co-EIF-1 and EIF-1*, but actively dissociates all the complexes in the presence of high Mg^{++} concentrations (5 mM) and low temperature.

We investigated the molecular properties of EIF-1* (Table II). EIF-1* is extremely heat stable and the solution retains full activity even after heating at 85° for 5 minutes. We routinely purify EIF-1* by phenol extraction. During phenol extraction, EIF-1* activity is found in the aqueous layer. However, EIF-1* activity is fully retained even after treatment with 0.3 M KOH for 24 hours at room temperature eliminating the possibility that EIF-1* is an oligoribonucleotide. EIF-1* activity is lost upon dialysis. Preliminary experiments with Sephadex (G-25) gel filtration indicated that EIF-1* has an approximate molecular weight of 2,500 (not shown here). Polyamines such as spermine and spermidine at different concentrations were also tested for EIF-1* activity. Both spermine (0.3 → 1 mM) and spermidine (0.5 → 3 mM) inhibit EIF-1 activity and the inhibition is observed both at 0 and 1 mM Mg^{++}. Treatment of EIF-1* with N-ethylmaleimide does not inhibit its stimulatory effect on Met-$tRNA_f$ binding to EIF-1 indicating that -SH group in EIF-1* is not involved in the stimulation of EIF-1 activity. EIF-1* activity is lost by treatment with high concentrations of pronase indicating that EIF-1* activity may be due to some low molecular weight protein.

EIF-1* was also found to be stimulatory to protein synthesis in reticulocyte lysate. For this experiment, we used micrococcal nuclease treated reticulocyte lysate as described by Pelham and Jackson (13) and studied the effect of addition of EIF-1* on exogeneously added hemoglobin mRNA-directed protein synthesis. This amino acid incorporating system very efficiently catalyzes [^{14}C] leucine incorporation and such incorporation is almost completely dependent on added hemoglobin mRNA (Fig. 2). Addition of EIF-1* consistently stimulated [^{14}C] leucine incorporation in this system (Fig. 2). However, the extent of such stimulation varied considerably with preparations

TABLE II

Effect of Different Treatments on EIF-1* Activity

EIF-1* Added	Treatment	[^{35}S] Met-tRNA$_f$ Bound to Millipore Filters (pmol)
-	-	0.8
+	-	3.6
+	Heat	3.2
+	Phenol	3.4
+	Alkali	3.4
+	Dialysis	0.8
+	NEM	3.3
+	Pronase (10 µg/ml)	3.2
+	Pronase (20 µg/ml)	2.9
+	Pronase (50 µg/ml)	1.6
+	Pronase (80 µg/ml)	0.9
+	[a]Pronase Control (80 µg/ml)	3.4
+	[b]Control	3.4

Standard Millipore filtration assay methods for [^{35}S] Met-tRNA$_f$ binding to EIF-1 in the presence of 1 mM Mg^{++} were used. EIF-1* (7.5 µg in 0.05 ml) was treated as follows and activities after each treatment were determined using 5 µl aliquots: (1) Heat treatment was at 85° for 5 minutes, (2) Phenol extraction procedure has been described under Materials and Methods, (3) Alkali treatment of EIF-1* was with 0.3 M KOH for 24 hours at room temperature and excess KOH was neutralized with 1 M HCl, (4) Dialysis of EIF-1* was against Buffer A containing 0.3 M KCl for 4 hours, (5) Preincubation with NEM (15 mM) was at 37° for 15 minutes and excess NEM was neutralized by adding 20 mM dithiothreitol, (6) Preincubation of EIF-1* with different concentrations of pronase was at 37° for 4 hours. Controls with different concentrations of pronase (without EIF-1*)[a] and EIF-1* (without pronase)[b] were run alongside. Protein concentrations of the factors used were: EIF-1, 1.7 µg; EIF-1*, 0.75 µg.

of lysate and EIF-1*. Addition of pure EIF-1 and Co-EIF-1 gave marginal stimulation of amino acid incorporation (not shown here). Some stimulation of amino acid incorporation however, was observed with EIF-2 (TDF) prepa-

ration presumably because of contamination of EIF-2 preparation with EIF-1* (not shown here). Polyamines such as spermine and spermidine at the concentration range $10^{-5} \rightarrow 10^{-3}$ M did not stimulate [^{14}C] leucine incorporation under the assay conditions and at higher concentrations were inhibitory to protein synthesis.

Discussion

It is now generally agreed that the Met-tRNA$_f$ binding factor (EIF-1) plays a key role in peptide chain initiation in eukaryotic cells (4). Previous work reported by our laboratory (1,2,8) and elsewhere (14,15) indicated that the activity of this factor is regulated by other proteins. At least, two other protein factors, Co-EIF-1 (1,8,15) and EIF-2 (TDF) (2,7) have been reported to control EIF-1 activity (1,2,7). These protein factors are also required to catalyze Met-tRNA$_f$ binding to 40S ribosomes. The heme-regulated inhibitor, HRI, phosphorylates the 38,000 subunit of EIF-1 (16-18) and the recent reports indicate that such phosphorylated EIF-1 may not be recognized by EIF-2 (TDF) (19,20) and also Co-EIF-1 (14,20) and is also inactive in the formation of the 40S initiation complex. In this paper, we present evidence that a heat stable low molecular weight protein factor is required for Met-tRNA$_f$ binding to EIF-1 in the presence of Mg^{++} (1 mM). The same EIF-1* preparation also inhibits Met-tRNA$_f$ binding to EIF-1 in the absence of Mg^{++} and stimulates protein synthesis *in vitro*. It is not apparent at present, how these activities are related to each other and whether all these three activities are due to the same or different factors.

Although the bulk of EIF-1* remains associated with the high molecular protein complex (EIF-2), a significant part of EIF-1* activity is also present in partially purified preparations of EIF-1 and is removed during further purification. Presumably, the conflicting reports in the literature concerning the Mg^{++} requirement for Met-tRNA$_f$ binding activity (6,9,10) may be explained by the presence of EIF-1* in the EIF-1 preparations used. As reported in this paper, the homogeneous preparations of EIF-1 are strongly inhibited by Mg^{++} (1 mM) and EIF-1* is necessary for restoring Met-tRNA$_f$ binding of EIF-1 at 1 mM Mg^{++}.

Several laboratories previously reported the presence of low molecular weight dialyzable factors which stimulate protein synthesis in reticulocyte lysate (21-24). One such low molecular weight factor was first reported as an oligoribonucleotide (21) but was later found to be alkali stable (22). The possibility that these low molecular weight factors are polyamines was also discussed (24). The characteristics of EIF-1* described in this paper have some resemblance to the low molecular weight factor(s) described by these authors (21-24). However, our present studies clearly establish that EIF-1* is neither an RNA nor a polyamine.

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References

1. Dasgupta, A., Das, A., Roy, R., Ralston, R., Majumdar, A., and Gupta, N. K. J. Biol. Chem. (in press).
2. Majumdar, A., Roy, R., Das, A., Dasgupta, A., and Gupta, N. K. (1977) Biochem. Biophys. Res. Commun. 78, 161-169.
3. Anderson, W. F. Bosch, L., Cohn, W. E., Lodish, H., Merrick, W. C., Weissbach, H., Wittman, H. G., and Wool, I. G. (1977) FEBS Letters 76, 1-10.
4. Weissbach, H., and Ochoa, S. (1976) Ann. Rev. Biochem. 45, 191-216.
5. Staehelin, T., Trachsel, H., Erni, B., Boschetti, A., and Schreier, M. H. (1975) Proceedings of the 10th FEBs Meeting p. 30.
6. Benne, R. Wong, C., Luedi, M., and Hershey, J. W. B. (1976) J. Biol. Chem. 251, 7675-7681.
7. Majumdar, A., Dasgupta, A., Chatterjee, B., Das, H. K., and Gupta, N. K. in Methods in Enzymology (Moldave, K. and Grossman, L., eds.), Academic Press, New York (in press).
8. Dasgupta, A., Majumdar, A., George, A. D., and Gupta, N. K. (1976) Biochem. Biophys. Res. Commun. 71, 1231-1241.
9. Schreier, M. H., Erni, B., and Staehelin, T. (1977) J. Mol. Biol. 116, 727-753.
10. Datta, A., DeHaro, C., Sierra, J. M. and Ochoa, S. (1977) Proc. Nat. Acad. Sci. U.S.A. 74, 1463-1467.
11. Gupta, N. K., Chatterjee, B., Chen, Y., and Majumdar, A. (1975) J. Biol. Chem. 250, 853-862.
12. Majumdar, A., Reynolds, S., and Gupta, N. K. (1975) Biochem. Biophys. Res. Commun. 67, 689-695.
13. Pelham, H., and Jackson, R. (1976) Eur. J. Biochem. 67, 247-257.
14. DeHaro, C., Datta, A., and Ochoa, S. (1978) Proc. Natl. Acad. Sci. U.S.A. 75, 243-247.
15. Malathi, V. G. and Majumdar, R. (1978) FEBS Letters 86, 155-159.
16. Farrel, P., Balkow, J., Hunt, T., Jackson, R. J. and Trachsel, H. (1977) Cell 11, 187-200.
17. Kramer, G., Cimadevilla, J. M. and Hardesty, B. (1976) Proc. Natl. Acad. Sci. U.S.A. 73, 3078-3082.
18. Levin, D. H., Ranu, R. S., Ernst, V., and London, I. M. (1976) Proc. Natl. Acad. Sci. U.S.A. 73, 3112-3116.
19. Das, A., and Gupta, N. K. (1977) Biochem. Biophys. Res. Commun. 78, 1433-1441.
20. Ranu, R. S., London, I. M., Das, A., Dasgupta, A., Majumdar, A., Ralston, R., Roy, R., and Gupta, N. K. (1978) Proc. Natl. Acad. Sci. U.S.A. 75, 745-749.
21. Bogdanovsky, D., Hermann, W., and Schapira, G. (1973) Biochem. Biophys. Res. Commun. 54, 25-32.
22. Berns, A., Salden, M., Bogdanovsky, D., Raymondjean, M., Schapira, G. and Bloemendal, H. (1975) Proc. Natl. Acad. Sci. U.S.A. 72, 714-718.
23. Shalden, M., Bisseling, T., Bern, A., and Bloemendal, H. (1975) Biochem. Biophys. Res. Commun. 65, 317-322.
24. Shalden, M. and Bloemendal, H. (1976) Biochem. Biophys. Res. Commun. 68, 157-161.
25. Krystosek, A., Cowther, M., and Kabat, D. (1975) J. Biol. Chem. 250, 6077-6084.