Restoration of Protein Synthesis in Lysed Rabbit Reticulocytes by the Enzymatic Removal of Adenosine 5'-Monophosphate with either AMP Deaminase or AMP Nucleosidase†

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ABSTRACT: Results are reported on the restoration of protein synthesis in lysed rabbit reticulocytes. We have demonstrated that (i) AMP endogenous to rabbit reticulocyte lysates and AMP added to lysates inhibit protein synthesis, (ii) protein synthesis can be restored when AMP is removed by AMP deaminase or AMP nucleosidase, (iii) an energy regenerating system is not needed for protein synthesis in lysed rabbit reticulocytes once AMP is removed, (iv) AMP inhibits protein synthesis without affecting the concentrations of GMP, ADP, GDP, GTP, or aminoacyl-tRNA, and (v) AMP inhibits peptide chain elongation. In unfractionated lysed rabbit reticulocytes supplemented with 1 mM ATP and 0.2 mM GTP, but without an energy regenerating system, there is little or no protein synthesis. The addition of either AMP nucleosidase (AMP → adenine + ribose 5-phosphate) or AMP deaminase (AMP → IMP) restores protein synthesis to rates 60% and 30%, respectively, of that observed in lysates incubated in the presence of an energy regenerating system. In lysates where adenylate kinase and AMP deaminase were inhibited with P^1,P^5-di(adenosine-5') pentaphosphate and coformycin, the addition of either 0.125 or 2 mM AMP to lysates containing an energy regenerating system inhibited protein synthesis by 12% and 82%, respectively. The inhibition of protein synthesis by 2 mM AMP did not change the concentration of either GDP or aminoacyl-tRNA. In lysates with an energy regenerating system, several purine nucleotide monophosphate analogues inhibited protein synthesis. AMP inhibits polypeptide chain elongation as determined by measuring the increase in ribosomes associated with polysomes and polyuridylic acid directed polyphenylalanine synthesis. The energy regenerating system in cell-free protein synthesis appears to be essential to maintain ATP and GTP concentrations and to remove inhibitory concentrations of AMP, ADP, and GDP.

A number of protein factors are required in peptide chain initiation, elongation, and termination (Caskey, 1980; Clark, 1980; Hunt, 1980). Investigations into these three processes have contributed to our knowledge of the regulation of protein synthesis. ATP, GTP, and an energy regenerating system are required for protein synthesis in cell-free systems (Kramer et al., 1976; Levin et al., 1973; Marcus, 1970; Merrick, 1979a; Ohta et al., 1967; Safer & Anderson, 1978; Traschel et al., 1977). The function of the energy regenerating system in cell-free protein synthesizing systems is to maintain ATP and GTP (Adamson et al., 1968; Rupniak & Quincey, 1975; Todd & Campbell, 1969; Zamecnik & Keller, 1954). Intracellular ATP depletion (20–30%) following anaerobic incubation markedly inhibits polypeptide synthesis in rabbit reticulocytes, ascites tumor cells, and rat liver (Freudenburg & Mager, 1971; Villa-Trevino et al., 1963). They concluded that it was unlikely that this small decrease in ATP could explain the decrease in the protein synthetic capacity of the cells. In vitro studies have suggested that AMP, ADP, and GDP are important in the regulation of protein synthesis. For example, AMP inhibits aminoacylation of tRNA (Malkin & Lipmann, 1969; Marshall & Zamecnik, 1970; Mitra & Mehler, 1966), ADP inhibits polypeptide elongation (Ibuki & Moldave, 1968) and eIF-2[1] phosphatase activity (Grankowski et al., 1980), and GDP inhibits ternary initiation complex formation (Walton & Gill, 1975, 1976), eIF-2 phosphatase activity (Safer & Jagus, 1979; Grankowski et al., 1980), and the elongation reactions of protein synthesis (Ibuki & Moldave, 1968; Lin et al., 1969; Henriksen et al., 1975; Jelenc & Kurland, 1979).

This laboratory has also reported on the stimulation and inhibition of protein synthesis in lysed rabbit reticulocytes by NAD^+, cAMP, sugar phosphates, and the low molecular weight inhibitors of translation, the 2',5'-oligo(adenylates) and the cordycepin analogue (Lennon et al., 1977; Wu et al., 1978a–c, 1979; Wu & Suhadolnik, 1980; Doetsch et al., 1981). The present study is an extension of these reports. Preliminary communications have appeared (Suhadolnik et al., 1979; Mosca et al., 1980).

Experimental Procedures

Materials. L-[U-^{14}C]Leucine (335 mCi/mmol), [8-^{14}C]-AMP (59 mCi/mmol), and L-[U-^{14}C]phenylalanine (521 mCi/mmol) were obtained from Amersham/Searle; creatine phosphate, creatine phosphokinase (ATP:creatine *N*-phosphotransferase, EC 2.7.3.2) (115 units/mg), pyruvate kinase (ATP:pyruvate phosphotransferase, EC 2.7.1.40) (385 units/mg), phosphoenolpyruvate, cycloheximide, poly(uridylic acid), phenylalanine-specific tRNA from brewers' yeast (type V), guanosine 5'-(β,γ-imidotriphosphate), ATP, ADP, 2',3',5'-AMP, IMP, CMP, UMP, 2'-dAMP, GTP, amino acids, and hemin (bovine, type I) were from Sigma Chemical Co.; Ap_5A, adenosine 5'-sulfate, GDP, and GMP were from Boehringer Mannheim; rabbit reticulocyte lysates were from Clinical Convenience; Centriflo membrane cones (type CF25) were from Amicon; density gradient grade sucrose (Ultra

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[1] Abbreviations: Ap_5A, P^1,P^5-di(adenosine-5') pentaphosphate; ara-AMP, 9-β-D-arabinofuranosyladenine 5'-monophosphate; Hepes, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; P-creatine, creatine phosphate; eIF-2, eukaryotic initiation factor 2; EF-1, eukaryotic elongation factor 1; EF-2, eukaryotic elongation factor 2.

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Pure) was from Schwarz/Mann; HPLC grade KH_2PO_4 was from Fisher Scientific. Coformycin was obtained from Dr. H. Umezawa; 3′-dGMP and 2′-amino-2′-dGMP were from Dr. F. Tomita. 3′-dAMP, ara-AMP, tubercidin 5′-monophosphate, toyocamycin 5′-monophosphate, and formycin 5′-monophosphate were synthesized, purified, and characterized according to Suhadolnik et al. (1977).

Enzyme Preparations. AMP nucleosidase (AMP phosphoribohydrolase, EC 3.2.2.4) (26.5 units/mg) was a generous gift from Dr. V. L. Schramm. AMP deaminase (AMP aminohydrolase, EC 3.5.4.6) (45 units/mg) was obtained from Sigma Chemical Co. The commercially available enzyme was concentrated from 6 to 200 units/mL before use with Centriflo membrane cones and addition of a stabilizing buffer to reduce the glycerol and KCl to 0.05% and 0.3 mM, respectively. The stabilizing buffer used was that of Agarwal & Parks (1977) at a KCl concentration of 230 mM, and mercaptoethanol was replaced by DL-dithiothreitol. The activity of the enzyme was determined by the change in the molar extinction coefficient of IMP and AMP; $\lambda_{235}^{pH6.3}$ was 3100. Assay mixtures contained 25 mM triethanolamine buffer (pH 7.0), 2 mM AMP, 45 mM KCl, 40 μM dithiothreitol, and 1 mM ATP. There was no loss of enzymatic activity for 3 days at 4 °C. Isolation of AMP deaminase from frozen rabbit muscle (obtained from Pel-Freeze) was as described by Smiley et al. (1967).

Assay of Protein Synthesis. Protein synthesis was assayed in rabbit reticulocyte lysates by [U-^{14}C]leucine incorporation into proteins modified as described (Wu et al., 1978a). The assay mixture was as follows: 50 μL of lysate, 20 mM Hepes (pH 7.6), 80 mM KCl, 1.5 mM $MgCl_2$, 1 mM ATP, 0.2 mM GTP, 50 μM solution of 19 amino acids (minus leucine), 65 μM [U-^{14}C]leucine (0.25 μCi), and 5 μM hemin, at a final volume of 60 μL. Where indicated, P-creatine (15 mM) and creatine phosphokinase (3 units/mL) were used as an energy regenerating system. Incubations were at 30 °C. Five-microliter aliquots were removed and processed as described (Wu et al., 1978a).

Preparation of Trichloroacetic Acid Soluble Nucleotides. Protein synthesis assays were terminated with 1 mL of 5% trichloroacetic acid at 4 °C and vortexed 1 min. The incubation mixture was centrifuged (8000*g*, 2 min), at 25 °C. The supernatants were treated 3 times with 4 mL of water-saturated diethyl ether to remove the trichloroacetic acid. The pH was adjusted to 9 by the addition of 15–20 μL of 1 N NH_4OH. Each sample was taken to dryness with a stream of filtered air. Recovery of radioactive material was greater than 90%. Thin-layer chromatography was performed by using Eastman cellulose sheets (no. 13254, with UV indicator) (solvent isobutyric acid/concentrated ammonia/water, 66:1:33 v/v/v). Standard nucleotides were chromatographed as markers during chromatography and visualized under UV light. R_f values for ATP, ADP, AMP, and IMP were 0.37, 0.46, 0.59, and 0.28, respectively. The chromatograms were cut into 1-cm^2 pieces, and the radioactivity was determined.

Polysome Distribution by Sucrose Density Gradient Centrifugation. Polysome distribution was determined as described (Safer et al., 1979).

Assay for Aminoacylation of tRNA in Unfractionated Lysed Rabbit Reticulocytes. The procedure of Andrulis & Arfin (1979) was modified to measure the amount of aminoacyl-tRNA present in lysate incubations. Lysates were incubated in 1 mL with a reaction mixture containing all of the components for protein synthesis with the exception of leucine. After a 5-min incubation, 10 μM [^{14}C]leucine (3.5 μCi) was added either alone or together with 550 μM Ap_5A, 60 μM coformycin, 10 μM cycloheximide, or 1–2 mM AMP. The assays were incubated for an additional 5 min, terminated, and processed as described (Andrulis & Arfin, 1979).

Assay for Poly(uridylic acid)-Directed Polyphenylalanine Synthesis. Polyphenylalanine synthesis was determined as described (Merrick, 1979b), using the rabbit reticulocyte aminoacyl-tRNA synthetase, phenylalanine-specific tRNA from brewers' yeast, 0.6 A_{260} unit of poly(uridylic acid), 0.4 A_{260} unit of sucrose cushion reticulocyte ribosomes, either 2 μg (reticulocyte) or 0.2 μg (50 units) (yeast) of EF-1, and either 0.2 μg (reticulocyte) or 100 units (yeast) of EF-2. Reticulocyte and yeast elongation factors were provided by Dr. W. Merrick and Dr. K. Chakraburty, respectively.

Radioactive Measurements. Radioactivity was measured by liquid scintillation spectrometry on a Beckman Model LS-100C scintillation spectrometer using formula 949 scintillation solution (New England Nuclear); efficiency for carbon-14 bound to filters was 42%, on Eastman cellulose sheets 75%, and in solution 90%.

Results

Restoration of Protein Synthesis in Lysed Rabbit Reticulocytes by the Enzymatic Removal of AMP in the Absence of an Energy Regenerating System. There is essentially no protein synthesis without a P-creatine/creatine phosphokinase energy regenerating system in lysed rabbit reticulocytes [Figure 1A (■)]. Similarly, there is no protein synthesis in lysates supplemented with 1–10 mM $MgATP^{2-}$ and 0.2–2 mM $MgGTP^{2-}$, without an energy regenerating system [Figure 1A (■)]. However, when the P-creatine/creatine phosphokinase energy regenerating system was added together with 1 mM ATP and 0.2 mM GTP, there was a linear incorporation of leucine into polypeptides [Figure 1A (●)]. Therefore, the energy regenerating system can restore protein synthesis. Because the energy regenerating system converts AMP, GMP, ADP, and GDP to ATP and GTP, it was reasoned that the inhibition of protein synthesis observed without an energy regenerating system might be due to endogenous concentrations of either AMP, GMP, ADP, or GDP. The possibility that the AMP in lysates, without an energy regenerating system, was inhibiting protein synthesis was tested experimentally by the enzymatic removal of AMP.

Addition of either AMP nucleosidase at 0.25, 0.5, and 1.0 unit [Figure 1A (△, ▲, ○)] or AMP deaminase at 0.1 and 0.3 unit [Figure 1B (▲, ○)] to lysates containing 90 μM endogenous AMP restored protein synthesis 60% and 30%, respectively. Adenine, ribose 5-phosphate, and IMP, the products of enzymatic hydrolysis of AMP, did not stimulate protein synthesis in lysates without an energy regenerating system [Figure 1 (■)]. To establish that the AMP nucleosidase and AMP deaminase were restoring protein synthesis by removing AMP, it was necessary to inhibit the enzymes. This was accomplished by the addition of either formycin 5′-monophosphate or coformycin. Formycin 5′-monophosphate is a known transition-state inhibitor of AMP nucleosidase (DeWolf et al., 1979); coformycin is a known potent inhibitor of AMP deaminase (Agarwal & Parks, 1977). Without an energy regenerating system, the addition of either 1 μM formycin 5′-monophosphate to AMP nucleosidase containing lysates or 42 μM coformycin to AMP deaminase containing lysates prevented the restoration of protein synthesis [Figure 1 (■)].

To prove that adenine was the product of AMP nucleosidase and IMP was the product of AMP deaminase, 60 μM [8-^{14}C]AMP (0.1 μCi) was added to lysates with 1 mM ATP, 0.2 mM GTP, and either 1 unit of AMP nucleosidase or 0.3

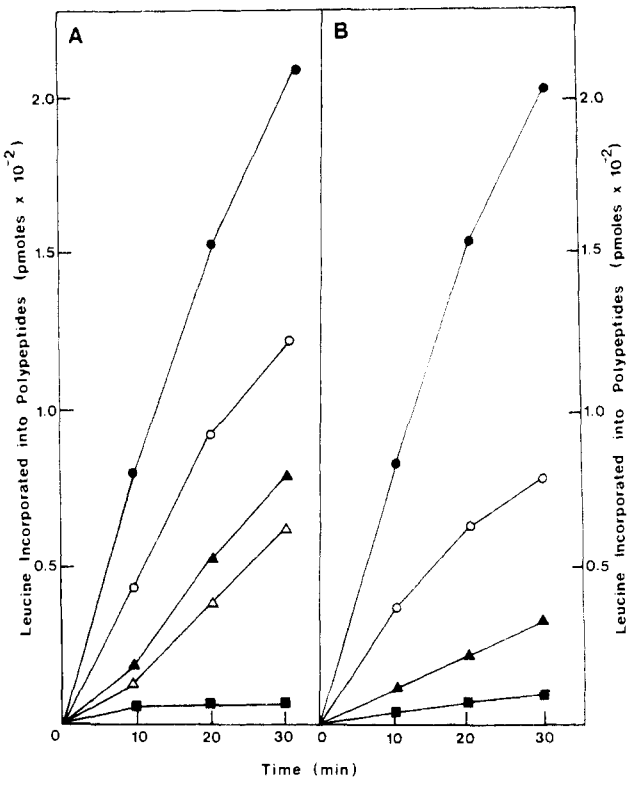

FIGURE 1: Restoration of protein synthesis by the enzymatic removal of AMP in lysed rabbit reticulocytes in the absence of an energy regenerating system. Protein synthesis was done as described under Experimental Procedures in either the presence or absence of AMP nucleosidase (panel A) or AMP deaminase (panel B). All protein synthesis assays contained 1 mM ATP and 0.2 mM GTP. (Panel A) (●) Lysates plus P-creatine/creatine phosphokinase; (△, ▲, and ○) 0.25, 0.5, and 1.0 unit of AMP nucleosidase, respectively (no energy regenerating system); (■) lysates without an energy regenerating system; (■) also represents four important control experiments, i.e., the addition of $MgATP^{2-}$ (1–10 mM) and $MgGTP^{2-}$ (0.2–2 mM), adenine and ribose 5-phosphate (0.1–1 mM), boiled enzyme, or AMP nucleosidase incubated with 1 μM formycin 5′-monophosphate. The concentration of formycin 5′-monophosphate for 95% inhibition of AMP nucleosidase was calculated by using the known catalytic constants ($K_{m,AMP}$ = 120 μM, $K_{i,FMP}$ = 0.04 μM) (DeWolf et al., 1979) and 100 μM for endogenous lysate AMP concentration. (Panel B) (●) Lysates plus P-creatine/creatine phosphokinase; (▲ and ○) 0.1 and 0.3 unit of AMP deaminase, respectively (no energy regenerating system); (■) lysates without an energy regenerating system; (■) also represents three important control experiments, i.e., the addition of IMP (0.1–1 mM), boiled enzyme, or AMP deaminase preincubated with 42 μM coformycin, 15 min, 30 °C.

unit of AMP deaminase. The lysates with AMP nucleosidase contained no [8-^{14}C]AMP following a 10-min incubation. Eighty percent of the carbon-14 was recovered as adenine, 12% as ATP, and 8% as adenosine. In the lysates with AMP deaminase, 5% of the carbon-14 was recovered as AMP, 30% as IMP, 40% as inosine, 20% as hypoxanthine, and 5% as ATP, after a 10-min incubation. The restoration of protein synthesis by the addition of AMP nucleosidase and AMP deaminase was observed in three lysate preparations purchased separately from Clinical Convenience (endogenous AMP concentrations were 0.09, 0.06, and 0.03 mM). AMP deaminase was isolated from two other sources (rabbit muscle and yeast) to show that the restoration of protein synthesis was not unique to the commercially available AMP deaminase. The restoration of protein synthesis by commercially available AMP deaminase was the same as that observed with AMP deaminase isolated from rabbit muscle or from yeast (data not shown).

Enzymatic Conversion of AMP to ATP, ADP, and IMP in the Presence of an Energy Regenerating System in Lysed Rabbit Reticulocytes. The addition of AMP nucleosidase to lysates incubated without an energy regenerating system not only decreased the concentration of AMP but also decreased the concentrations of ADP and GDP (Table II, experiments 2 and 3). Therefore, the affect of AMP on protein synthesis with an energy regenerating system was studied to show that the restoration of protein synthesis by AMP nucleosidase and AMP deaminase was due to the removal of AMP from the lysate. The addition of 1 mM AMP to lysates with an energy regenerating system inhibited protein synthesis by 20% (data not shown); however, 90% of the AMP was converted to ATP in 3 min. Because of the rapid conversion of AMP to ATP with an energy regenerating system, it was necessary to inhibit the conversion of AMP to ATP and AMP to IMP. This was accomplished by the addition of Ap_5A and coformycin to the lysates. Ap_5A is an inhibitor of adenylate kinase (Purich & Fromm, 1972), and coformycin is an inhibitor of AMP deaminase (Agarwal & Parks, 1977). The inhibition of adenylate kinase by Ap_5A decreased the conversion of AMP to ATP from 91% to 30% and increased AMP from 0.5% to 8%; however, 56% of the AMP was deaminated to IMP (Table I, experiments 1 and 3). The addition of 60 μM coformycin together with 550 μM Ap_5A to the lysates decreased the deamination of AMP to IMP from 56% to 4% (experiments 3 and 4). The combination of Ap_5A and coformycin decreased the conversion of AMP to ATP from 91% to 57% and increased AMP from 0.5% to 36% (experiments 1 and 4). When 0.1–1 mM AMP was added to lysates containing Ap_5A and coformycin, the AMP increased from 43% to 70% (experiments 5–8).

Restoration of Protein Synthesis in Lysed Rabbit Reticulocytes following the Enzymatic Removal of AMP in the Presence of an Energy Regenerating System. In protein-synthesizing assays containing P-creatine/creatine phosphokinase plus or minus coformycin, the incorporation of leucine into polypeptide was linear [Figure 2 (●)]. Although there was no inhibition of protein synthesis by coformycin, there was a 22% inhibition of protein synthesis when Ap_5A was added to the lysates (○). The inhibition of protein synthesis was 82% when 2 mM AMP was added together with Ap_5A and coformycin (■). AMP was removed by the addition of AMP nucleosidase 5 min after the addition of AMP to show that AMP was inhibiting protein synthesis; protein synthesis was restored to a rate almost equivalent to that observed in control lysates (□). The addition of 1 or 2 mM adenine and 1 or 2 mM ribose 5-phosphate, the products of AMP hydrolysis by AMP nucleosidase, together with 2 mM AMP did not restore polypeptide synthesis (■). AMP at 0.125, 0.25, 0.5, and 1 mM inhibited protein synthesis 12, 23, 44, and 62%, respectively, in assays containing P-creatine/creatine phosphokinase, Ap_5A, and coformycin (data not shown).

Purine Nucleotide Concentrations in Lysates As Determined by High-Performance Liquid Chromatography. Walton & Gill (1979) reported that changes in the adenylate pool affect the molar concentrations of GMP, GDP, and GTP in vitro. Therefore, it was necessary to determine the affect of adding AMP to lysates on the concentrations of AMP, GMP, ADP, GDP, ATP, and GTP. This was accomplished by high-performance liquid chromatography. In lysates containing 1 mM ATP, 0.2 mM GTP, and an energy regenerating system, there was an 18-, a 0-, a 30-, an 11-, and a 6-fold decrease in the concentrations of AMP, IMP, GMP, ADP, and GDP, respectively (Table II, experiments 2 and 4). When Ap_5A and coformycin were added to the P-creatine/creatine phosphokinase containing lysates, the concentration of AMP increased

Table I: Changes in AMP, ADP, ATP, and IMP in Lysed Rabbit Reticulocytes in the Presence of an Energy Regenerating System, Ap_5A and Coformycin[a]

		AMP		ADP		ATP		IMP	
expt	addition	dpm	%	dpm	%	dpm	%	dpm	%
1	none	250	0.5	500	1	45 500	91	4000	8
2	coformycin	250	0.5	500	1	48 000	96	1000	2
3	Ap_5A	4 000	8	500	1	15 000	30	28000	56
4	Ap_5A and coformycin	18 000	36	500	1	27 400	57	2000	4
5	Ap_5A, coformycin, and 0.1 mM AMP	21 500	43	1000	2	24 000	48	2500	5
6	Ap_5A, coformycin, and 0.25 mM AMP	2 300	46	1000	2	21 500	43	3000	6
7	Ap_5A, coformycin, and 0.5 mM AMP	28 500	57	500	1	16 500	33	2500	5
8	Ap_5A, coformycin, and 1 mM AMP	35 000	70	1000	2	10 000	20	3000	6

[a] All assays (60 μL) were performed as described under Experimental Procedures in the presence of P-creatine/creatine phosphokinase, 1 mM ATP, and 0.2 mM GTP. After 5-min incubation at 30 °C, 30 μM [8-^{14}C]AMP (60 μCi/μmol, 0.1 μCi) was added to all incubations. Coformycin (60 μM, to inhibit endogenous AMP deaminase) (experiments 2 and 4–8) and hemin (5 μM) were added to thawing lysates. Ap_5A (550 μM) (experiments 3–8) and AMP at the concentrations indicated (experiments 5–8) were added to lysate incubations after 5 min at 30 °C at the same time as [8-^{14}C]AMP. Assays were terminated at 10 min with 1 mL of 5% trichloroacetic acid at 4 °C and further processed as described under Experimental Procedures. The percent conversion of nucleotides was based on ^{14}C in the isolated nucleotides; 50 000 dpm of the trichloroacetic acid soluble nucleotides was added to the Eastman cellulose sheets.

Table II: Purine Nucleotide Concentrations in Lysates As Determined by High-Performance Liquid Chromatography[a]

									protein synthesis	
expt	addition	AMP (mM)	IMP (mM)	GMP (mM)	ADP (mM)	GDP (mM)	ATP (mM)	GTP (mM)	restored (%)	inhibited (%)
1	endogenous to lysate	0.090	0.07	0.020	0.210	0.070	0.200	0.030		
2	none	0.090	0.060	0.030	0.440	0.090	1.100	0.300		97
3	1 unit of AMP nucleosidase	0.010	0.040	<0.001	0.070	0.025	0.950	0.400	60	
4	P-creatine/creatine phosphokinase	0.005	0.060	<0.001	0.040	0.015	1.550	0.350	100	
5	expt 4 plus Ap_5A and coformycin	0.040	0.040	<0.001	0.030	0.015	1.520	0.350		23
6	expt 5 plus AMP (2 mM)	1.600	0.250	<0.001	0.030	0.015	1.750	0.350		82
7	expt 6 plus 1 unit of AMP nucleosidase	0.010	0.230	<0.001	0.030	0.010	1.560	0.340	64	
8	expt 4 plus AMP (2 mM)	0.005	0.040	<0.001	0.120	0.020	3.550	0.400		30

[a] Assays (120 μL), experiments 2–8, were performed as described under Experimental Procedures in the presence of 1 mM ATP and 0.2 mM GTP. Experiment 1 shows the nucleotide concentrations endogenous to the lysate. Experiment 2 is the nucleotide concentrations for lysates incubated without an energy regenerating system. Experiment 3 is the nucleotide concentrations for lysates incubated with 1 unit of AMP nucleosidase (no energy regenerating system). Experiments 4–8 are the nucleotide concentrations for lysates incubated with an energy regenerating system and either coformycin (60 μM), Ap_5A (550 μM), AMP (2 mM), or AMP nucleosidase (1 unit) as indicated. Coformycin was added to the thawing lysates to inhibit endogenous AMP deaminase; Ap_5A and AMP were added to the lysates after a 5-min incubation at 30 °C; AMP nucleosidase was added to the assays 5 min after the addition of Ap_5A and AMP. A 20-μL aliquot was removed from each assay and incubated with [U-^{14}C]leucine (0.1 μCi) for protein synthesis determination. The incorporation of [^{14}C]leucine was reported as either the percent restoration or the percent inhibition of protein synthesis. The assay incubations (experiments 2–6 and 8) were terminated after 10 min at 30 °C with an equal volume of 10% trichloroacetic acid at 4 °C by a modification of Jacobson et al. (1979). Experiment 7 was terminated 5 min after the addition of AMP nucleosidase. Extraction of nucleotides was done by a 1-min vortexing and incubating at 4 °C for 30 min. The precipitated proteins were centrifuged (8000*g*, 4 °C) and discarded. The supernatants were extracted 4 times with 1 mL of water-saturated diethyl ether. The ether was evaporated under a stream of filtered air. Samples were neutralized with 5 μL of 1.6 M Tris-HCl buffer, pH 7.0. Analysis was performed on a Waters Associates high-performance liquid chromatography unit, Model 2210 with a Partisil-10 SAX column (Whatman). Ten-microliter samples were injected. Displacing buffer was the following: 0.01 M KH_2PO_4 (pH 4.0) for AMP, IMP, and GMP; 0.25 M KH_2PO_4 (pH 4.0) for ADP and GDP; 0.6 M KH_2PO_4 for ATP and GTP; flow rate 2 mL/min. Retention times for AMP, IMP, GMP, ADP, GDP, ATP, and GTP were 9, 11.5, 17, 6, 7.5, 4, and 4.5 min, respectively. Calculation of concentrations of nucleotides was based on peak area. Areas under the peak were determined by multiplying the height of the peak by the width at half-height (Brown, 1970). Peaks were identified by injecting known standards together with the sample.

from 5 to 40 μM, whereas the concentrations of GMP, IMP, ADP, GDP, ATP, and GTP did not change significantly (experiments 4 and 5). When either 2 mM AMP or 2 mM MgAMP was added with Ap_5A and coformycin to lysates, the concentration of AMP increased from 0.04 to 1.6 mM; IMP and ATP concentrations increased slightly, while GMP, ADP, GDP, and ATP concentrations did not change (experiments 5 and 6). The addition of AMP nucleosidase to lysates inhibited by 2 mM AMP to which Ap_5A and coformycin had been added decreased the concentration of AMP from 1.6 to 0.01 mM (experiments 6 and 7). Under these conditions, protein synthesis was restored to 64% of controls [Figure 2 (□)]. The role of the inhibitors Ap_5A and coformycin in the maintenance of AMP concentrations in lysates was demonstrated by the observation that 2 mM AMP was completely converted to ATP within 5 min (experiment 8). This is in contrast to 80% of the 2 mM AMP remaining as AMP in lysates to which Ap_5A and coformycin were added (experiment 6).

Effect of Purine Nucleotide Analogues on Protein Synthesis in Lysed Rabbit Reticulocytes. Purine nucleotide analogues were used to determine the structural requirements of AMP for the inhibition of protein synthesis. The purine nucleotides (1 mM) were added to lysates in the presence of Ap_5A and

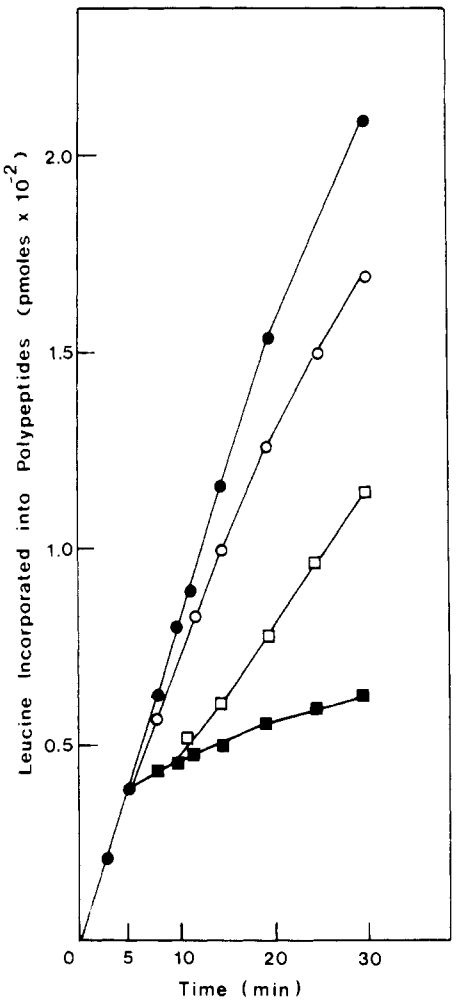

FIGURE 2: Restoration of protein synthesis in lysed rabbit reticulocytes by the enzymatic removal of AMP in the presence of an energy regenerating system. Sixty-microliter assays were incubated as described under Experimental Procedures in the presence of P-creatine/creatine phosphokinase and contained 1 mM ATP and 0.2 mM GTP. Coformycin (60 μM) was added to the thawing lysates to inhibit endogenous AMP deaminase; Ap_5A (550 μM) was added to inhibit endogenous adenylate kinase, and AMP or MgAMP (2 mM) was added to the lysates after a 5-min incubation at 30 °C. AMP nucleosidase was added to the assays 10 min after lysate incubation (5 min after the addition of Ap_5A and AMP). (●) With or without coformycin; (○) either Ap_5A or Ap_5A plus coformycin; (■) AMP plus Ap_5A and coformycin; (□) 1 unit of AMP nucleosidase plus AMP, Ap_5A, and coformycin; (■) also represents a necessary control where adenine and ribose 5-phosphate (1–2 mM) were added with AMP, Ap_5A, and coformycin.

coformycin to inhibit endogenous adenylate kinase and AMP deaminase. When either the 2′- or 3′-hydroxyl group of AMP was replaced by a hydrogen (i.e., 2′-dAMP or 3′-dAMP), the inhibition of protein synthesis was less than with AMP (Figure 3). When the ribosyl moiety of AMP was replaced with D-arabinosyl moiety (i.e., ara-AMP), the inhibition of protein synthesis was the same as in lysates with the same concentration of AMP. When the monophosphate was on C-2′ or C-3′ (i.e., 2′-AMP or 3′-AMP), there was no inhibition of protein synthesis. When the 5′-phosphate of AMP was replaced with a 5′-sulfate (i.e., adenosine 5′-sulfate), the inhibition of protein synthesis increased (Figure 3). IMP in the presence of an energy regenerating system inhibits protein synthesis, whereas CMP, UMP, GMP, 3′-dGMP, and 2′-amino-2′-dGMP did not. The 5′-monophosphates of the two naturally occurring nucleoside antiobiotics toyocamycin and formycin inhibited protein synthesis, but tubercidin 5′-monophosphate was not an inhibitor.

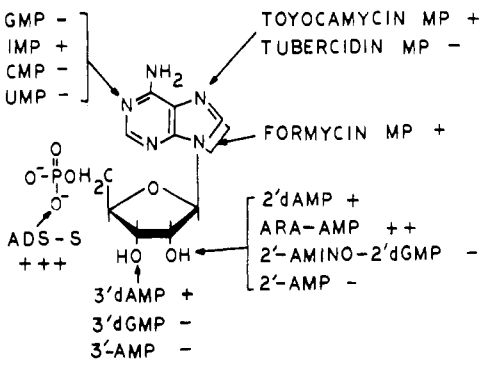

FIGURE 3: Summary of the effect of structural modifications in AMP on protein synthesis in lysed rabbit reticulocytes. Lysates were incubated in the presence of Ap_5A (550 μM) and coformycin (60 μM) to inhibit endogenous adenylate kinase and AMP deaminase. The nucleotides [at 1 mM concentrations plus 1 mM $Mg(OAc)_2$] were incubated for 30 min as described under Experimental Procedures. All assays were incubated in the presence of P-creatine/creatine phosphokinase and contained 1 mM ATP and 0.2 mM GTP. The data are presented as a comparison to the inhibition of protein synthesis observed by AMP. (+) Inhibition of protein synthesis was less than with AMP; (++) inhibition of protein synthesis was equivalent to with AMP; (+++) inhibition of protein synthesis was greater than with AMP; (−) no inhibition of protein synthesis.

Table III: Effect of Structural Modifications in AMP on Poly(uridylic acid)-Directed Polyphenylalanine Synthesis[a]

	inhibition of polyphenylalanine synthesis (%)					
	rabbit reticulocyte elongation factors (mM)			yeast elongation factors (mM)		
addition	0.05	0.5	1	0.05	0.5	1
none	0	0	0	0	0	0
5′-AMP	25	51	53	40	56	57
toyocamycin 5′-phosphate		55	96		41	80
adenosine 5′-sulfate		9	0		0	0
GMP		0	0		0	0
$MgGDP^{2-}$		0	0		0	0
IMP		0	0		0	9
AMP plus 1 unit of AMP nucleosidase		0			0	

[a] Assays (50 μL) were incubated at 37 °C as described under Experimental Procedures. All compounds were added at the start of the incubation.

Effect of AMP on Polysomes in Lysed Rabbit Reticulocytes. For determination of the site of inhibition of the protein synthesizing machinery by AMP, the polysome distribution in reticulocyte lysates was studied by sucrose density gradient centrifugation (Figure 4). The polysome profiles after 10-, 20-, and 30-min incubations were analyzed in lysates containing Ap_5A and coformycin with or without 1 mM AMP. When lysates were incubated with 1 mM AMP, there was an increase in the amount of ribosomes associated with polysomes. There was also a shift from smaller to larger polysomes (Figure 4). When AMP was not added to lysates, the addition of either Ap_5A or coformycin had no effect on the polysome distribution.

Effect of AMP on Poly(uridylic acid)-Directed Polyphenylalanine Synthesis. The polysome distribution in lysates incubated with AMP suggests that polypeptide chain elongation is primarily inhibited (Figure 4). For investigation of the effect of AMP on the elongation process of protein synthesis, poly(uridylic acid)-directed polyphenylalanine synthesis

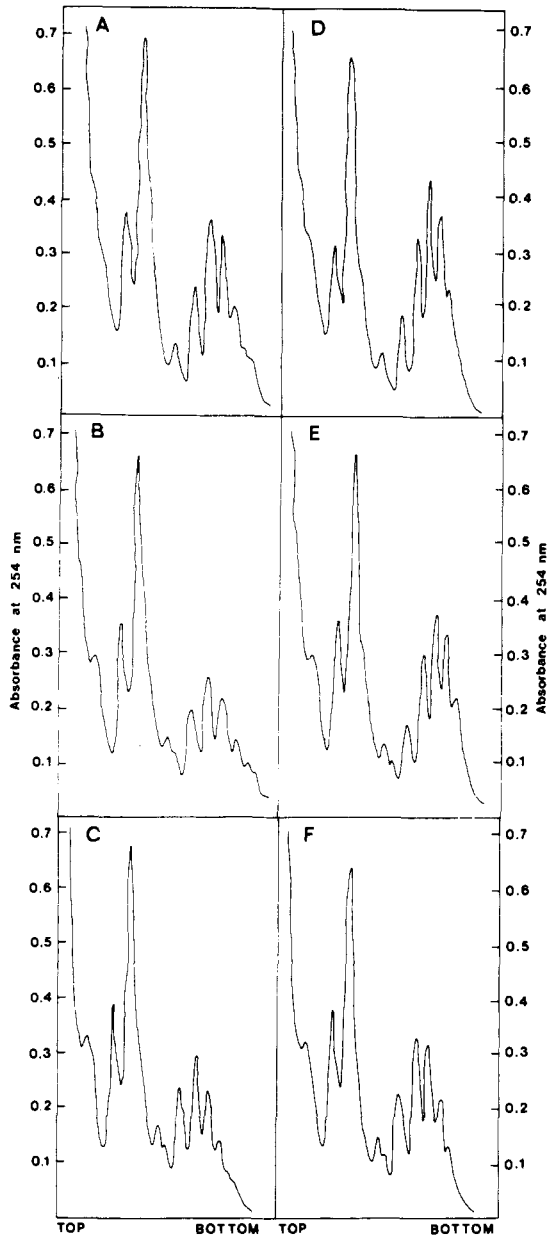

FIGURE 4: Effect of AMP on polysomes in lysed rabbit reticulocytes. Lysates (120 μL) were incubated as described under Experimental Procedures in the presence of P-creatine/creatine phosphokinase, 1 mM ATP, and 0.2 mM GTP. Coformycin (60 μM), Ap_5A (550 μM), and AMP (1 mM) were added as described (see legend to Figure 2). At 5, 15, and 25 min after the addition of either Ap_5A or Ap_5A plus AMP, 100-μL aliquots were diluted in 300 μL of 10 μM guanosine 5'-(β,γ-imidotriphosphate) at 4 °C. The distribution of polysomes was determined by sucrose density gradient centrifugation as described under Experimental Procedures. (A, B, and C) Minus AMP, plus or minus Ap_5A, and coformycin; (D, E, and F) plus AMP, Ap_5A, and coformycin. Incubations were for (A and D) 10, (B and E), 20, and (C and F) 30 min.

was studied at 10 mM Mg^{2+} (Merrick, 1979b). These assays measure polypeptide elongation independent of polypeptide initiation. When 0.05 mM AMP was added, polyphenylalanine synthesis was inhibited 25 and 40%, respectively, with rabbit reticulocyte and yeast elongation factors (Table III). When the AMP concentration was increased to 0.5 and 1 mM, the inhibition of polyphenylalanine synthesis was 55% at both concentrations with rabbit reticulocyte and yeast elongation factors (Table III). As in the inhibition of protein synthesis by AMP in unfractionated lysates, when AMP nucleosidase was added, there was no inhibition of polyphenylalanine synthesis (Table III). For determination of the structural requirements of AMP needed to inhibit polyphenylalanine synthesis, AMP was replaced by purine nucleotide analogues in the elongation assays. Only toyocamycin 5'-monophosphate inhibited polyphenylalanine synthesis (Table III). The inhibition of the elongation process by toyocamycin 5'-monophosphate was similar to the inhibition observed in unfractionated rabbit reticulocyte lysates (Figure 3). When AMP was replaced by either adenosine 5'-sulfate, GMP, $MgGDP^{2-}$, or IMP (1 mM), which inhibited the incorporation of leucine into protein in unfractionated rabbit reticulocyte lysates incubated with an energy regenerating system 85, 9, 7, and 16%, respectively, polyphenylalanine synthesis was not inhibited (Table III).

Effect of AMP on Aminoacylation of tRNA in Unfractionated Lysed Rabbit Reticulocytes. Because Malkin & Lipmann (1969), Marshall & Zamecnik (1970), and Mitra & Mehler (1966) have reported that 0.1 mM AMP inhibits the aminoacylation of tRNA by using partially purified enzyme preparations, the effect of AMP on aminoacyl-tRNA synthetase in unfractionated rabbit reticulocyte lysates was studied. In cycloheximide-inhibited lysates containing P-creatine/creatine phosphokinase and the addition of either coformycin, coformycin plus Ap_5A, or coformycin plus Ap_5A and 1 or 2 mM AMP, the amount of $Leu\text{-}tRNA^{Leu}$ formed was 24, 26, 26, and 27 pmol, respectively.

Discussion

We have demonstrated that protein synthesis can be restored in reticulocyte lysates by the enzymatic removal of AMP that is either endogenous or exogenous to lysates. The restoration of protein synthesis is more pronounced following the addition of AMP nucleosidase in which AMP is converted to adenine and ribose 5-phosphate than that observed following the addition of AMP deaminase in which AMP is converted to IMP. The data we obtained clearly show that the addition of 1 mM ATP and 0.2 mM GTP to lysates without an energy regenerating system, where the concentration of AMP is 90 μM, will not restore protein synthesis unless the AMP is removed enzymatically. We demonstrated that one site of inhibition of the protein synthesizing machinery by AMP is polypeptide elongation as determined by polysome profiles and polyphenylalanine synthesis. The specific step in polypeptide elongation where AMP inhibits protein synthesis is not known. AMP may indirectly affect EF-1 or EF-2 catalytic activity, possibly via ribosomal subunit phosphorylation. Our results led us to conclude that AMP does not increase the concentration of GDP (Table II, experiments 4 and 6), nor does AMP decrease the concentration of $Leu\text{-}tRNA^{Leu}$. The AMP-dependent hydrolysis of aminoacyl-tRNA was reported by Mitra & Mehler (1966) to require AMP and PP_i. When they added pyrophosphatase, the hydrolysis of the aminoacyl bond in the aminoacyl-tRNA was not affected by AMP. Therefore, the discrepancy between the observed AMP inhibition of aminoacylation of the partially purified aminoacyl-tRNA synthetase and the aminoacyl-tRNA synthetase in the unfractionated lysates might be explained by pyrophosphatase activity in the reticulocyte lysates. In addition, depletion of Mg^{2+} by either Ap_5A or increased concentrations of ATP could not explain the observed inhibition of protein synthesis by AMP because (i) the addition of Ap_5A to lysates only inhibits protein synthesis by 22%, whereas the addition of AMP with Ap_5A inhibits protein synthesis by 82% (Figure 2), (ii) the replacement of AMP by MgAMP results in a similar inhibition of protein synthesis to that observed with AMP, and (iii) the addition of AMP nucleosidase to AMP-inhibited lysates rapidly re-

stored protein synthesis. These results are in agreement with our observation that protein synthesis is restored when AMP is removed from lysates (Figure 1 and Table II, experiments 6 and 7).

The possibility that GMP, ADP, and GDP would also inhibit protein synthesis was considered. We used high-performance liquid chromatography to determine the concentrations of purine nucleotides in lysates without an energy regenerating system (Table II, experiment 2). Whereas there was no protein synthesis in lysates in which the concentrations of AMP, GMP, ADP, and GDP were 90, 30, 440, and 90 μM, respectively, protein synthesis could be restored following the addition of 1 unit of AMP nucleosidase (Table II, experiment 3). Of interest is the observation that the polysome profiles are maintained in lysates incubated without an energy regenerating system, where there is no protein synthesis. This result led us to conclude that the inhibition of protein synthesis in lysates without an energy regenerating system occurs at the polypeptide elongation step. This observsation is consistent with the inhibition of the elongation process by AMP (Figure 4).

The structural requirements for AMP inhibition of protein synthesis and polyphenylalanine synthesis were determined by (i) replacing the purine ring with a pyrimidine ring, (ii) modifying the imidazole ring, (iii) modifying the amino group on carbon 6 of adenine, (iv) modifying the ribosyl moiety of AMP, (v) changing the position of the monophosphate on the ribosyl ring, and (vi) changing the anionic group at carbon 5′ (Figure 3). It is apparent that the purine ring and the ribosyl moiety can be modified and still inhibit protein synthesis. Furthermore, replacement of the phosphate anion with a sulfate anion at carbon 5′ of adenosine inhibits protein synthesis. However, when the phosphate group is on either carbon 2′ or carbon 3′ of adenosine, there is no inhibition of protein synthesis. In contrast to the inhibition of protein synthesis in unfractionated lysates by AMP and adenosine 5′-sulfate, the inhibition of elongation as determined by polyphenylalanine synthesis allows modification in the imidazole ring but requires the amino group on carbon 6 of adenine and a phosphate as the anionic group on carbon 5′ (Table III). The differences in the effect on protein synthesis observed with the analogues tested indicate that polypeptide elongation may not be the only site of inhibition by AMP. In support of this statement, our earlier studies reported the stimulation of protein synthesis in lysed rabbit reticulocytes by NAD^+ (Lennon et al., 1977; Wu et al., 1978a,c). The mechanism of NAD^+ stimulation of protein synthesis was correlated to the generation of ATP via activation of glycolysis. As reported here, protein synthesis in the absence of an energy regenerating system is not limited by the concentrations of ATP or GTP, but by AMP, ADP, and GDP. Therefore, it now seems reasonable to conclude that the stimulation of protein synthesis observed by NAD^+ was due to the removal of AMP and ADP through adenylate kinase and substrate phosphorylation. Continued phosphorylation of AMP through the adenylate kinase reaction was demonstrated by the increase in the adenylate energy charge from 0.7 to 0.93 (Lennon et al., 1977). Because the stimulation of protein synthesis by NAD^+ has been shown to affect initiation complex formation (Wu et al., 1978a,c), it is also possible that AMP may affect protein synthesis initiation. In addition to the possible regulation of protein synthesis by AMP as described here and the regulation of protein synthesis by GDP as described by Safer & Jagus (1979), Gonzatti-Haces & Traugh (1982) have recently reported that the phosphorylation of eIF-2α by hemin and the phosphorylation of eIF-2β by 2,3-diphosphoglyceric acid could regulate their respective protein kinases in vivo and may also regulate protein synthesis.

The results reported here also clearly define the requirement of the energy regenerating system in protein synthesis. The energy regenerating system is essential for the conversion of AMP to ATP. If AMP is not removed from lysates, even though the concentrations of added ATP and GTP are 1 and 0.2 mM, respectively, AMP will inhibit protein synthesis (Figures 1 and 2). In addition, the energy regenerating system is also necessary to maintain the concentrations of ATP and GTP for protein synthesis. The restoration of protein synthesis by the enzymatic removal of AMP eliminates the need for creatine phosphokinase as an energy regenerating system (Figure 1). Because commercial samples of creatine kinase are known to be contaminated with nucleases (Ehrenfeld & Brown, 1981), the elimination of this enzyme in protein-synthesizing lysates could circumvent this potential problem.

In view of our knowledge with respect to the regulation of cellular processes by AMP, i.e., purine biosynthesis in which AMP is a feedback inhibitor (Wynggarden & Ashton, 1959), glycogen synthesis and degradation which is regulated by AMP (Yeh et al., 1980; Stalmans et al., 1971), glycolysis and gluconeogenesis in which AMP stimulates phosphofructokinase and inhibits fructose diphosphatase (Ramaiah, 1974), and lipogenesis in which AMP regulates acetyl-CoA carboxylase (Yeh et al., 1980), it is tempting to suggest that changes in the concentration of AMP could affect protein synthesis. It is worth noting that protein synthesis is inhibited during nutritional deprivation and anoxia. Under these conditions, there are marked changes in the cellular concentrations of AMP, ADP, and ATP. For example, in Ehrlich tumor cells, isolated rat heart cells, liver cell suspensions, and intact rabbit reticulocytes, protein synthesis was inhibited following either nutritional deprivation or anoxia (Edwards et al., 1979; Freudenburg & Mager, 1971; Henderson et al., 1977; Jefferson et al., 1971; Live & Kaminskas, 1975; Panet & Atlan, 1979; van Venrooij et al., 1970, 1972). Under these experimental conditions, the concentrations of cellular AMP increased 3–8-fold while the concentrations of cellular ATP decreased 0.2–4-fold. Furthermore, several laboratories have reported a reduced rate of protein synthesis together with decreased ratios of ATP:AMP in muscle from adenosine deaminase deficiency disease (Fishbein et al., 1978; Sanada & Yamaguchi, 1979; Petryshyn & Nicholls, 1976, 1978; Farrell & Olson, 1973; Sabina et al., 1980; Buchwald et al., 1981). However, further studies are required in intact mammalian cells and tissues to test the inhibitory effects of AMP on protein synthesis and to clarify the physiological importance of AMP regulation.

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Registry No. AMP nucleosidase, 9025-45-0; AMP deaminase, 9025-10-9; AMP, 61-19-8; GMP, 85-32-5; IMP, 131-99-7; CMP, 63-37-6; UMP, 58-97-9; 3′-dAMP, 15731-72-3; 3′-dGMP, 6220-62-8; 3′-AMP, 84-21-9; toyocamycin 5′-monophosphate, 15742-78-6; tubercidin 5′-monophosphate, 16719-46-3; formycin 5′-monophosphate, 13270-66-1; 2′-dAMP, 653-63-4; ara-AMP, 29984-33-6; 2′-amino-

2′-dGMP, 72189-87-8; 2′-AMP, 130-49-4; MgGDP, 7277-99-8; adenosine 5′-sulfate, 2304-12-3.

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A Collagenolytic Serine Protease with Trypsin-like Specificity from the Fiddler Crab *Uca pugilator*†

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ABSTRACT: A second collagenolytic serine protease has been isolated from the hepatopancreas of the fiddler crab, *Uca pugilator*. This enzyme cleaves the native triple helix of collagen under physiological conditions of pH, temperature, and ionic strength. In addition to its collagenolytic activity, the enzyme exhibits endopeptidase activity toward other polypeptides and small molecular weight synthetic substrates. The polypeptide bond specificity of this enzyme is similar to that of bovine trypsin as is its interaction with specific protease inhibitors. The amino-terminal sequence of this enzyme displays significant homology with other serine proteases, most notably with that of crayfish trypsin, and demonstrates that this enzyme is a member of the trypsin family of serine endopeptidases. The relatively unique action of this protease with regard to both collagenous and noncollagenous substrates has important implications concerning the specificity and mechanism of collagen degradation.

The trypsin-related serine proteases comprise one of the best characterized families of proteins which have evolved from a common ancestor. Serine proteases from many sources display a marked degree of similarity in primary structure (Woodbury et al., 1978) and share common elements of three-dimensional structure and mechanism of action (Blow et al., 1969; Shotton & Watson, 1970; Stroud et al., 1971). Despite these similarities, it is clear that serine proteases function in a variety of diverse and often highly specific physiological roles. The most well studied of these are the enzymes from vertebrate systems which participate in such processes as digestion, blood coagulation, fertilization, and the immune response. Although serine proteases from invertebrate sources have been known for some time, they are less well studied, and their specific functions have been less well-defined. Recently, a new group of invertebrate serine proteases, which possess the ability to cleave the native triple helix of collagen under physiological conditions of pH, temperature, and ionic strength, has become recognized. These enzymes are the first serine proteases known to possess collagenolytic activity and as such represent a new group of collagenases. However, unlike the metalloenzyme collagenases of mammals, these invertebrate collagenases are involved in digestion rather than morphogenesis.

Collagenolytic serine proteases have been reported in the fungus *Entomophthora coronata* (Hurion et al., 1979), the insect *Hypoderma lineatum* (Lecroisey et al., 1979), and the fiddler crab, *Uca pugilator* (Eisen & Jeffrey, 1969; Eisen et al., 1970, 1973). The first to be reported and the most extensively characterized of these is crab protease I from the fiddler crab. Its complete amino acid sequence has been determined and shown to possess significant homology to the trypsin-related serine proteases of mammals (Grant et al., 1980). Furthermore, its specificity with both collagenous and noncollagenous substrates has been documented (Grant & Eisen, 1980; Grant et al., 1982; Welgus et al., 1982). Crab protease I successfully degrades all five types of native collagen and is a good general protease of noncollagenous polypeptides. The enzyme appears to be a chymotrypsin-like enzyme in that it displays a significant degree of preference for phenylalanyl, tryptophanyl, and leucyl residues although it has also been shown to cleave at both positively and negatively charged residues as well. It is specifically inhibited by chymostatin while leupeptin is without effect.

In addition to crab protease I, the hepatopancreas of the fiddler crab contains at least one additional distinct collagenolytic serine protease. The present study details the purification and characterization of crab protease II and demonstrates that its specificity, at least with respect to noncollagenous substrates, is markedly different from that of crab protease I and is very similar to that of bovine trypsin.

Materials and Methods

N-Benzoyl-L-valylglycyl-L-arginine *p*-nitroanilide (BzValGlyArgNA), *N*-benzoyl-L-tyrosine ethyl ester (BzTyrEE), N^{α}-*p*-tosyl-L-lysine chloromethyl ketone hydrochloride (TLCK), *N*-benzyloxycarbonyl-L-phenylalanine chloromethyl ketone (ZPCK), and bovine insulin were from Sigma Chemical Co. *N*-benzoyl-L-arginine *p*-nitroanilide (BzArgNA) was from Vega Biochemicals. Bovine trypsin was a product of Worthington Biochemical Corp., and all sequencing reagents were from Beckman. All other reagents were of the highest grade commercially available.

Bovine insulin oxidized chain B was produced from bovine insulin by performic acid oxidation (Weber et al., 1972) and separated from the oxidized A chain by high-pressure liquid chromatography on a Varian Model 5020 liquid chromatograph. A Beckman ultrasphere octadecyl reverse-phase column (4.6 × 250 mm) was used with a linear gradient of 1-propanol (1%/min) in 15 mM tris(hydroxymethyl)aminomethane hydrochloride (Tris-HCl), pH 7.1 at 30 °C. The proteolytic products of crab protease II digestion of oxidized insulin chain B were separated in the same manner.

Proteolytic digests of polypeptide substrates were performed in 0.05 M Tris buffer, pH 8.0, at room temperature. Enzyme

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