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Deformylation and Protein Biosynthesis*

David M. Livingston and Philip Leder

ABSTRACT: An enzyme which removes formyl groups from *N*-formylmethionylaminoacyl-transfer ribonucleic acid and its chemically synthesized analog, *N*-formylmethionylpuromycin, has been prepared from extracts of *Escherichia coli*. The specificity of the enzyme is that expected of a deformylase active during protein biosynthesis. It fails to act upon *N*-formylmethione or *N*-formylmethionyl-transfer ribonucleic acid until the blocked amino acid is transferred into an initial intermediate of protein synthesis, *N*-formylmethionylaminoacyl-transfer ribonucleic acid. The critical role of the formyl blocking group prior to the formation of the initial peptide bond and its presumed redundancy thereafter are consistent with this specificity. As previously demonstrated and confirmed in these studies, the enzyme also deformylates free formyl peptides. In addition, the differences observed in N terminals of *in vitro* and *in vivo* synthesized *E. coli* and certain bacteriophage proteins are likely accounted for by the marked lability of the enzyme in crude and purified extracts. Both SO_4^{2-} and SO_3^{2-} increase the initial reaction rate of the enzyme, but neither of these anions nor a variety of other compounds prevent the enzyme's rapid *in vitro* inactivation. Following the removal of the formyl blocking group, N-terminal methionine is removed by an aminopeptidase present in these preparations, a reaction required by the nature of the N-terminal residues of many bacterial and ribonucleic acid viral proteins.

The amino blocking group of F-Met-tRNA[1] is required for efficient formation of the initial ribosome binding complex and first peptide bond during protein synthesis (Bretscher and Marcker, 1966; Eisenstadt and Lengyel, 1966; Nakamoto and Kolakofsky, 1966; Zamir *et al.*, 1966; Leder and Nau, 1967; Salas *et al.*, 1967). Studies using synthetic RNA messengers, however, suggest that such blocking groups are not involved in subsequent steps in the elaboration of the polypeptide chain (Nakamoto and Kolakfsky, 1966), although they may serve to protect the nascent peptide from aminopeptidase degradation (Bretscher and Marcker, 1966). The ultimate redundancy of the formyl group is indicated by its absence in completed *Escherichia coli* and RNA bacteriophage proteins (Waller, 1963; Konigsberg *et al.*, 1966), despite its universal occurrence in those proteins synthesized *in vitro* (Adams and Capecchi, 1966; Capecchi, 1966; Webster *et al.*, 1966). An enzymatic activity which catalyzes the removal of formyl groups from free formyl peptides has, in fact, been detected in extracts of both *E. coli* B and *Bacillus stearothermophilus* (Gussin *et al.*, 1966; Fry and Lamborg, 1967; Adams, 1968). If, however, the formyl group is not involved in the elaboration of the polypeptide chain, it is reasonable to expect F-Met to serve as a substrate for such an enzyme *only after* its incorporation into the initial F-Met-aminoacyl-tRNA intermediate during protein synthesis. These studies present an additional test of the redundancy of the formyl group during protein assembly by using F-

* From the Laboratory of Biochemistry, National Cancer Institute, National Institutes of Health, and the Department of Health, Education, and Welfare, Bethesda, Maryland 20014. *Received September 9, 1968.*

[1] Abbreviations: F-Met, *N*-formylmethionine; F-Met-Puro, *N*-formylmethionylpuromycin; $AUGU_3$ and $AUGU_6$, the oligoribonucleotides corresponding to the trinucleoside diphosphate ApUpG followed by a sequence of three and six uridylic acid residues, respectively.

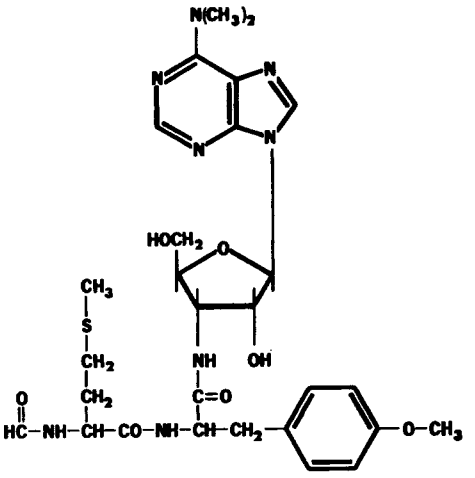

FIGURE 1: Structural formula of F-Met-puromycin.

Met-Phe-tRNA and its analog, F-Met-puromycin (Figure 1), as substrates for a partially purified deformylase which we have prepared from *E. coli*. By removing the formyl blocking group only after formation of the initial peptide bond, this enzyme exhibits the specificity expected of a deformylase which is active during protein biosynthesis.

Materials and Methods

Materials. [^{14}C]Formic acid (specific activity 21.8–45.6 μCi/μmole) was from Nuclear Chicago Corp., L-[^{14}C]methionine and L-[^{14}C]leucine (specific activity 293 and 275 μCi/μmole, respectively) from New England Nuclear Corp.; L-[^{13}C] amino acids were purchased from Mann Research Laboratories. Dicyclohexylcarbodiimide was from Distillation Products. Norit A was from Pfanstiehl Laboratories. Bovine pancreatic deoxyribonuclease was from Worthington Biochemicals. [5,10-^{14}C]Methenyltetrahydrofolate (specific activity 62.3 μCi/μmole) was the generous gift of Dr. Herbert Dickerman, and highly purified, endopeptidase-free hog kidney leucine aminopeptidase was kindly given to us by Dr. S. R. Himmelhoch. Components of the protein synthetic system were supplied by Dr. R. W. Erbe and Mrs. M. M. Nau, to whom we are most grateful.

Synthesis, Purification, and Characterization of Substrates. RADIOACTIVE FORMYLAMINO ACIDS. Radioactive *N*-formylamino acids were prepared from [^{14}C]- and [^{13}C]formic acid and the corresponding [^{13}C]- or [^{14}C]-amino acid by the method of Sheehan and Yang (1958). Blocked amino acids were separated from unblocked starting material by passage of the entire reaction mixture, after dilution with 100 volumes of H_2O (total volume 1.0–2.0 ml), through a 1.3 × 1.5 cm column of Dowex 50-H^+, previously equilibrated with 1 N HCl and washed to pH 5.0 with H_2O immediately prior to use. The formylamino acids were eluted with H_2O until no further radioactivity appeared in the effluent. The pooled eluates were dried several times under reduced pressure to remove formic and acetic acids and identified by their electrophoretic mobility at pH 3.5 (see below) as compared to authentic standards.

RADIOACTIVE FORMYLAMINOACYLPUROMYCIN. [^{14}C]-Formylamino acid or formyl-[^{14}C]amino acid (1 equiv dissolved in dioxane) was treated with puromycin dihydrochloride (1.5 equiv dissolved in chloroform–dimethylformamide, 8:1 v/v), previously neutralized with 3.0 equiv of triethylamine, in the presence of dicyclohexylcarbodiimide (1 equiv in methylene chloride). The reaction was carried out for 24 hr at 23° in the dark. The radioactive product was isolated by Whatman No. 3MM paper electrophoresis in 0.05 M ammonium formate (pH 3.5) for 1 hr at 60 V/cm. The paper was dried at room temperature and the product was identified as an ultraviolet-light-absorbing spot adjacent to an authentic standard. The area was cut out, eluted with H_2O, dried several times under reduced pressure, and finally dissolved in H_2O and stored at −23° at pH 6.0 with negligible deformylation over 6 months. Yields were from 25 to 70%.

An ultraviolet spectrum of the compound in 0.1 N NaOH was identical with that of puromycin with maxima and minima of 275 and 244 mμ, respectively. A spectrum in 1.0 N HCl was also identical with that of puromycin under the same conditions. The formylaminoacylpuromycins were fully resistant to highly purified, hog kidney leucine aminopeptidase (Himmelhoch and Peterson, 1968) until hydrolysis in 0.1 M HCl at 37° for 15 hr. The enzyme was able to release radioactive amino acid from the acid-deformylated compounds. Mild acid hydrolytic products of *N*-formyl-[^{13}C]Met-puromycin or *N*-formyl-[^{14}C]Leu-puromycin had the electrophoretic mobilities in 1.0 M pyridine acetate (pH 3.5) or 0.05 M ammonium formate (pH 3.5) expected of [^{14}C]Met-puromycin and [^{14}C]Leu-puromycin, respectively. Both formylaminoacylpuromycins were >90% hydrolyzed to their respective deformylated analogs after incubation for 15 hr in 1.0 N HCl at 37°. The structural formula for F-Met-puromycin is shown in Figure 1.

[^{14}C]F-MET-tRNA. The preparation of [^{14}C]F-Met-tRNA has been described previously (Leder and Bursztyn, 1966) and differed only in that [5,10-^{14}C]methenyltetrahydrofolate (specific activity 62.3 μCi/μmole) was used as formyl donor in the transformylase reaction.

F-MET-[^{14}C]PHE. F-Met-[^{14}C]Phe was synthesized enzymatically and isolated from protein synthetic reactions directed by the defined mRNA, $AUGU_8$, by the method of Erbe and Leder (1968).

Assays. DEFORMYLATION OF [^{14}C]F-MET-PUROMYCIN. Reaction mixtures, unless otherwise indicated, contained 0.02 M sodium phosphate (pH 7.2) or 0.02 M Tris-Cl (pH 7.5), 1.39 M $(NH_4)_2SO_4$, 7.3 × 10^{-2} mM [^{14}C]F-Met-Puro (specific activity 1.7–2.0 μCi/μmole), and enzyme, as indicated, in a total volume of 0.05 ml. Incubation was at 23° for 10 min or as indicated. The reaction was stopped by the addition of 1.0 ml of a 1% w/v aqueous suspension of Norit A (acid-washed activated charcoal). The tube was mixed vigorously and then poured over a nitrocellulose filter (Millipore, HAWP, 0.45 μ) fitted in a multiple filtration apparatus

(Leder and Byrne, 1964). The filters were washed once with 1.0 ml of H_2O and both filtrate and wash collected directly in a scintillation vial. Triton X–toluene–2,5-diphenyloxazole–1,4-bis[2-(5-phenyloxazolyl)]benzene scintillation fluid (10 ml) (Patterson and Greene, 1965) was added and the samples were counted in a scintillation counter. The appearance of radioactivity in the filtrate is a direct measure of the release of [^{14}C]formate from the puromycin derivative, puromycin being quantitatively adsorbed to charcoal. Characterization of the degradation product is noted below. All reactions were performed in duplicate and appropriate quench corrections applied.

DEFORMYLATION OF [^{14}C]F-MET-PHE- AND [^{14}C]F-MET-PHE-PHE-tRNAs. The defined mRNA-directed protein synthetic system of Erbe and Leder (1968) was used for the synthesis of F-Met-Phe- and F-Met-Phe-Phe-tRNAs. For this, the mRNAs are $AUGU_3$ and $AUGU_6$, respectively. Each complete 0.05-ml synthetic reaction mixture contained 0.05 M Tris-acetate (pH 7.2); 0.05 M NH_4Cl; 5 mM magnesium acetate; 0.04 mM oligoribonucleotide; 1 mM GTP; 1.5 and 3.7 μg of initiation factors F_1 and F_2, respectively (Stanley *et al.*, 1966); 24 and 11 μg of transfer factors T and G, respectively (Nishizuka and Lipmann, 1966); 0.3 A_{260} seven-times-NH_4Cl-washed *E. coli* MRE 600 ribosomes (Erbe and Leder, 1968); 15 $\mu\mu$moles of [^{14}C]F-Met-tRNA (plus 20 other [^{12}C]aminoacyl-tRNAs). Synthetic incubation was at 23° for 30 min and resulted in the synthesis of approximately 2 $\mu\mu$moles of F-Met-Phe- or F-Met-Phe-Phe-tRNA in the complete system. In the absence of transfer factor G, the product directed by both mRNAs is approximately 2 $\mu\mu$moles of F-Met-Phe-tRNA. The tubes were chilled and to each was added 37.5 μg of partially purified deformylase containing 69.5 μmoles of $(NH_4)_2SO_4$ in a volume of 0.025 ml. Incubation was resumed for 10 min at 23° following which the tubes were again chilled and 1.0 ml of ice-cold 10% trichloroacetic acid was added to each. After vigorous mixing, the suspensions were poured over nitrocellulose filters (Millipore, HAWP, 0.45 μ) and washed with 1.0 ml of 10% trichloroacetic acid. The entire filtrate was collected in a scintillation vial to which 10 ml of Triton X–toluene–2,5-diphenyloxazole–1,4-bis[2-(5-phenyloxazolyl)]benzene was added. The appearance of radioactivity in the filtrate corresponded to the release of [^{14}C] formate from tRNA. Characterization of the degradation product is noted below.

Electrophoretic Analysis of Products of the Deformylase Reaction. Reactions were carried out as noted above, but were stopped by the addition of 0.28 ml of 0.20 M $Ba(OH)_2$ with the formation of a ($BaSO_4$) precipitate. The suspensions were mixed thoroughly and centrifuged for 5 min at 2000 rpm in an International refrigerated centrifuge. The soluble products were applied to Whatman No. 3MM paper, dried under cooled air or nitrogen, and then electrophoresed in 1 M pyridine acetate (pH 3.5) at 60 V/cm for 35–40 min. Electrophoretic lanes were cut into 1-cm strips which were placed in scintillation vials containing 10 ml of toluene–2,5-diphenyloxazole–1,4-bis[2-(5-phenyloxazolyl)]benzene scintillation fluid and counted. All samples were corrected for intrinsic absorption of radioactivity on paper. Authentic standards were detected by ultraviolet light absorption, ninhydrin, or, in the case of blocked amino acids and peptides, by the method of Bachur (1965).

TABLE I: Preparation of Deformylase.[a]

Fraction	Total Protein (mg)	Sp Act. (units/mg of protein)	Total Act. (units × 10^{-3})
Crude extract	650	23	15
60% $(NH_4)_2SO_4$ precipitate	585	11	6.4
60% $(NH_4)_2SO_4$ supernatant	50	49.5	2.5

[a] Components of reaction mixtures are as described in Materials and Methods. Incubation was for 1 min at 23°. A unit is defined as the release of 1 mμmole of [^{14}C]formate from [^{14}C]F-Met-puromycin/min at 23°.

Preparation of Deformylase. Frozen *E. coli* MRE-600 (Cammack and Wade, 1965) was thawed at 23° and suspended in an equal weight of 0.05 M sodium phosphate (pH 7.2) at 0° and disrupted in a prechilled French pressure cell at 15,000 psi. The extract was diluted with an equal volume of the same buffer and deoxyribonuclease was added to a final concentration of 20 μg/ml of extract. The mixture was then incubated at 23° for 4 min and immediately chilled. All subsequent steps were carried out at 0–4°. The extract was then centrifuged at 48,000*g* for 15 min and the supernatant was collected and centrifuged at 78,000*g* for 30 min. The upper seven-eighths of the resulting supernatant fluid was removed and to it was added a saturated solution of $(NH_4)_2SO_4$ to 60% saturation. The precipitate was collected by centrifugation at 48,000*g* for 15 min. The supernatant was frozen and stored in liquid nitrogen for use as a partially purified deformylase fraction. Under these conditions there was no appreciable loss of activity of the frozen enzyme for at least 8 weeks. Protein concentration was determined by the method of Lowry *et al.* (1951).

Results

Preparation and Characteristics of the Enzyme. The most prominent and complicating characteristic of this deformylase is its extraordinary instability under a wide variety of conditions (*cf.* Figure 5 and Adams (1968)). This property has, unfortunately, prevented extensive purification of the enzyme. Nevertheless, the enzyme has been separated from others which act on free F-Met. Over 40% of the deformylase activity is lost during a 60% $(NH_4)_2SO_4$ fractionation of crude, ribosome-free, bacterial extract (Table I). This activity is not regained by reconstituting complementary pre-

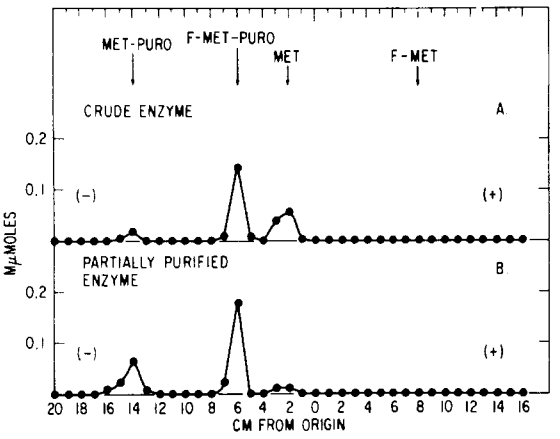

FIGURE 2: Effect of crude and partially purified deformylase on the degradation of F-[^{14}C]Met-puromycin. Conditions of incubation, components of reaction mixtures, and details of electrophoretic analysis are as described in Materials and Methods. F-[^{14}C]Met-puromycin (specific activity 7.2 μCi/μmole) (0.54 mμmole) was present in each reaction; 52 μg (1.1 units) of crude extract protein (A) and 22.5 μg (1.1 units) of partially purified enzyme (B) were used.

cipitate and supernatant fractions. The 60% $(NH_4)_2SO_4$ supernatant fraction contains approximately 8% of the protein originally present in the crude extract and approximately 28% of the surviving activity. The enzyme's striking instability does not permit meaningful comparison of specific activities during purification. Because of its specificity, demonstrated below, the 60% $(NH_4)_2SO_4$ supernatant was the partially purified deformylase preparation used in these studies. Dialysis, Sephadex G-25

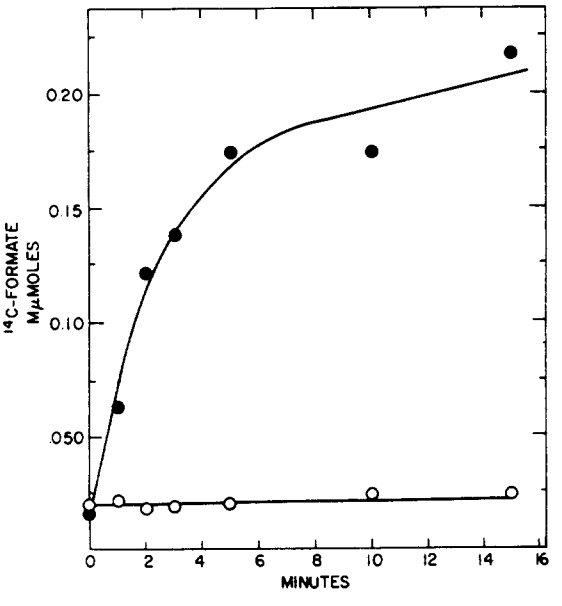

FIGURE 3: Time dependence of the deformylase reaction. Conditions of incubation and components of reaction mixtures are as described in Materials and Methods. The final concentration of $(NH_4)_2SO_4$ was 1.56 M. Partially purified deformylase protein (3.75 μg) was added to each reaction mixture. (●——●) Reaction mixtures containing partially purified enzyme. (○—○) Heat-inactivated enzyme (5 min at 100°).

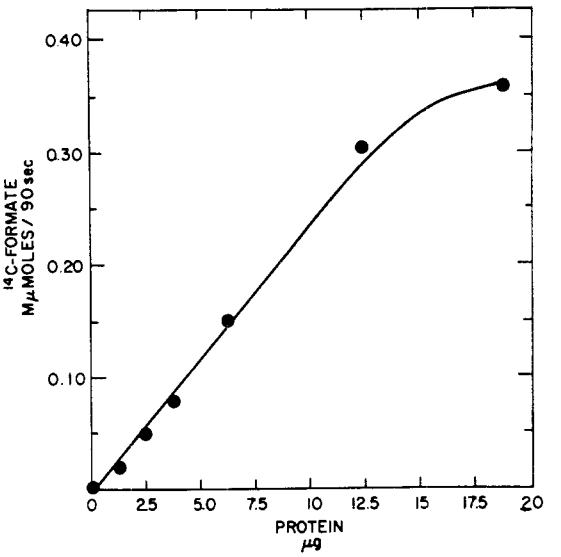

FIGURE 4: Dependence of initial reaction rate upon enzyme concentration. Components of reaction mixtures are as described in Figure 3 and in Materials and Methods. Incubation was for 90 sec at 23°.

and G-100, and DEAE-cellulose column chromatography under a wide variety of conditions resulted in rapid inactivation of the enzyme. The activity could not be restored by reconstituting high and low molecular weight components nor by complementing fractions with one another or with rate-limiting amounts of active or inactivated crude extracts.

The ability of both crude and partially purified enzyme to deformylate F-Met-puromycin, an analog of F-Met-aminoacyl-tRNA, is shown by an electrophoretic analysis of a reaction mixture utilizing F-[^{14}C]Met-puromycin as substrate. The predominant product of the degradation of F-Met-puromycin by crude enzyme containing 1.1 units of deformylase activity (Figure 2A) is free methionine (95 μμmoles) accompanied by a small amount of Met-puromycin (13 μμmoles). Degradation with the partially purified enzyme, containing the same amount of deformylase activity, leads to the accumulation of Met-puromycin (88 μμmoles) with very little production of free methionine (11 μμmoles). The crude enzyme contains an aminopeptidase activity which is largely although not completely removed by $(NH_4)_2SO_4$ fractionation. Thus, the degradative sequence is likely initiated by the deformylation reaction with subsequent release of free methionine from the Met-puromycin intermediate.

The kinetics of the deformylation reaction at 23° using [^{14}C]F-Met-puromycin as substrate are shown in Figure 3. The reaction proceeds in a linear fashion for approximately 2 min and is complete in 10 min. The initial reaction rate is linearly dependent upon enzyme concentration (Figure 4) and all studies of the effects of heat and activators on the enzyme were carried out at enzyme concentrations in this range of linear initial rate response. The extraordinary instability of the enzyme is evidenced by its inactivation at 32 and 37° (Figure 5). The half-life of the enzyme at 37° is 60 sec; at 32°,

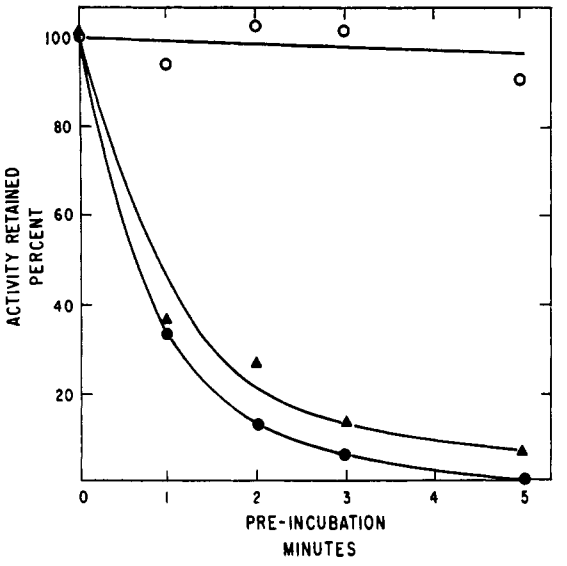

FIGURE 5: Heat inactivation of deformylase. Components of reaction mixtures are described in Materials and Methods. Partially purified deformylase (4.8 μg) was used in each reaction mixture. In addition, reaction mixtures contained sodium phosphate (pH 7.2) (1.2 mM) and $(NH_4)_2SO_4$ (1.56 M). Each reaction mixture was preincubated at the indicated temperature (closed circles, 37°; closed triangles, 32°; open circles, 0°) for the indicated time in the absence of substrate and cooled to 0°. Deformylation reactions were initiated by the addition of substrate and subsequent incubation was for 90 sec at 23°.

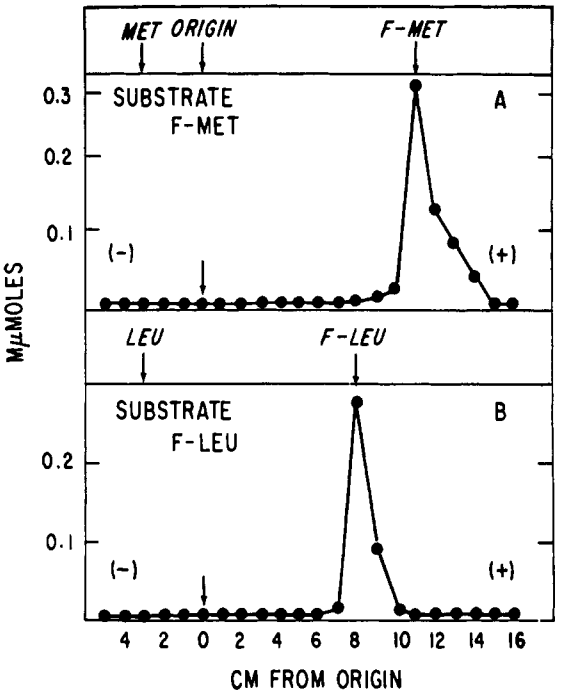

FIGURE 6: Effect of deformylase on F-[^{14}C]Met and F-[^{14}C]-Leu. Conditions of incubation, components of reaction mixtures, and details of electrophoretic analysis are as described in Materials and Methods. In addition, reaction mixtures contained sodium phosphate (pH 7.2) (0.02 M), partially purified deformylase (37.5 μg), and the appropriate F-[^{14}C]-amino acid (0.50 mμmole) (specific activity 7.2 μCi/μmole).

70 sec. A variety of compounds, potential cofactors and substrates, were tested in an effort to inhibit heat inactivation but were uniformly unsuccessful.[2]

Specificity and Role of the Enzyme in Protein Synthesis. As shown above, a crude bacterial extract and the deformylase which was prepared from it readily deformylate F-Met-puromycin, an analog of one of the first intermediates in bacterial protein synthesis (*cf.* Figure 1; Yarmolinsky and de la Haba, 1959). Such an activity is consistent with the redundancy of the formyl group in the subsequent steps of polypeptide elongation. If deformylase is active during protein biosynthesis, one would expect its activity to be restricted to intermediates in this process which no longer require the blocking group. In contrast with nonspecific deacylases (Birnbaum *et al.*, 1952; Vogel, 1953; Ohmura and Hayaishi, 1957; Fry and Lamborg, 1967; Weissbach and Redfield, 1967), this partially purified enzyme does not deformylate free F-Met (Figure 6A) or F-Leu (Figure 6B). F-Met-tRNA, the blocking group of which is required for efficient initiation of protein synthesis (Bretscher and Marcker, 1966; Eisenstadt and Lengyel, 1966; Nakamoto and Kolakofsky, 1966; Zamir *et al.*, 1966; Leder and Nau, 1967; Salas *et al.*, 1967), is not deformylated or deacylated by the partially purified enzyme. There is no significant difference between the amount of formate released from [^{14}C]F-Met-tRNA in the presence of active enzyme (0.87 μμmole) as compared with that released in the presence of heat-inactivated enzyme (0.82 μμmole). In addition, the enzyme more efficiently removes formate from F-Met-Puro than F-Leu-Puro. Electrophoretic analyses of the degradation products of both substrates (Figure 7) indicate an accumulation of only 88 μμmoles (Figure 7B) of [^{14}C]Leu-Puro compared with 474 μμmoles of [^{14}C]Met-Puro (Figure 7A), both formylated substrates being present in equimolar amounts at the same specific radioactivity.

The ability of the enzyme to deformylate preferentially F-Met-puromycin and its inability to deformylate F-Met and F-Met-tRNA are consistent with deformylation occurring only after transfer of F-Met to an oncoming aminoacyl-tRNA and formation of the initial peptide bond during protein synthesis. That F-Met is incorporated into F-Met-aminoacyl-tRNA prior to deformylation and that this reaction can occur immediately after formation of the first peptide bond is illustrated by an experiment in which release of formate is dependent upon the $AUGU_3$- and $AUGU_6$-directed syntheses of F-Met-Phe- and F-Met-Phe-Phe-tRNAs (Table II). Although residual polypeptide synthetic activity is associated with highly purified ribosomes (Pestka, 1968), Erbe and Leder (1968) have shown that synthesis of the dipeptide F-Met-Phe is very largely dependent upon, among other components, the presence of the appropriate mRNA and the transfer factor T of Nishizuka and Lipmann (1966) which is involved in binding of

[2] Components used in heat inactivation studies were as follows: $(NH_4)_2SO_4$, $MgCl_2$, Met, F-Met, F-Met-puromycin, sucrose, glycerol, albumin, dithiothreitol, EDTA, 5-formyltetrahydrofolate, *E. coli* MRE-600 ribosomes, *E. coli* B tRNA, and yeast extract.

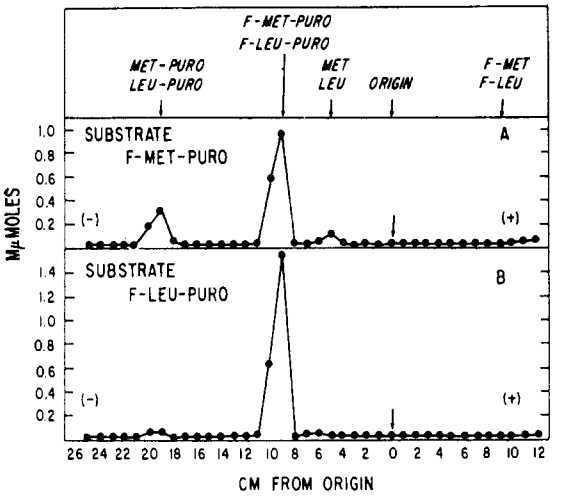

FIGURE 7: Deformylation of F-[^{14}C]Met-puromycin and F-[^{14}C]Leu-puromycin. Conditions of incubation, components of reaction mixtures, and details of electrophoretic analysis are as described in Materials and Methods. In addition, reaction mixtures contained sodium phosphate (pH 7.2) (0.016 M), $(NH_4)_2SO_4$ (0.84 M), F-[^{14}C]Met-puromycin or F-[^{14}C]-Leu-puromycin (2.6 mμmoles) (specific activity 0.72 μCi/μmole), and partially purified deformylase (22.5 μg).

aminoacyl-tRNA to ribosomes. Another transfer factor, G of Nishizuka and Lipmann (1966), is not required for dipeptide synthesis, but is required for translation of subsequent codons, a process presumed to involve translocation of both mRNA, and peptidyl-tRNA (Erbe and Leder, 1968). As shown in Table II, deformylation is also dependent upon elements required for the incorporation of F-Met into F-Met-Phe-tRNA, namely, transfer factor T, ribosomes, and mRNA, but independent of transfer factor G which is required for translation of the third codon of the $AUGU_6$ message and, hence, synthesis of the tripeptide F-Met-Phe-Phe. In this case, both complete reactions and those minus transfer factor G resulted in the synthesis of approximately 2.0 μμmoles of F-Met-peptidyl-tRNA. The deformylation product of an $AUGU_3$-directed synthetic reaction in which Phe

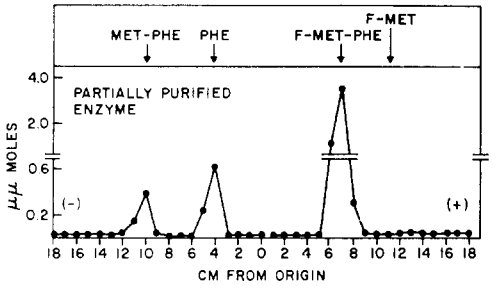

FIGURE 8: Deformylation of F-Met-[^{14}C]Phe. Components of reaction mixtures and details of electrophoretic analysis are as described in Materials and Methods. In addition, each reaction mixture contained 37.5 μg of partially purified active or heat-inactivated (5 min at 100°) deformylase and approximately 6.5 μμmoles of F-Met-[^{14}C]Phe. Incubation was for 10 min at 23°. Although not shown here, the reaction with heat-inactivated deformylase did not yield significant amounts of Met-[^{14}C]Phe or [^{14}C]Phe.

TABLE II: Dependence of Deformylase-Catalyzed Formate Release upon Incorporation of F-Met into F-Met-peptidyl-tRNA.[a]

System	[^{14}C]Formate Released (μμmoles)
mRNA: $AUGU_3$	
Complete	0.79
Minus transfer factor G	1.24
Minus transfer factor T	0.29
Minus transfer factors G and T	0.19
Minus ribosomes	0
mRNA: $AUGU_6$	
Complete	1.23
Minus transfer factor G	1.18
Minus transfer factor T	0.27
Minus transfer factors G and T	0.25
Minus ribosomes	0.10
mRNA: None	
Complete	0

[a] The reaction mixture components and conditions of incubation are as described in Materials and Methods. The experiment involved a synthetic preincubation during which the appropriate [^{14}C]F-Met-peptidyl-tRNA was, where possible, synthesized, followed by incubation in the presence of deformylase. In each case, a control value of released radioactivity determined with heat-inactivated deformylase has been subtracted from that obtained with active enzyme. This value averaged 1.27 ± 0.20 μμmoles.

was radioactive has been characterized electrophoretically after its release by alkaline hydrolysis from tRNA and is, as expected, the unblocked dipeptide, Met-Phe.

Although both F-Met-Phe-tRNA and F-Met-puromycin serve as substrates for the deformylation reaction, Adams (1968) and Fry and Lamborg (1967) have shown that free peptides are deformylated as well. The deformylase which we have prepared is also active in this respect. This is illustrated by an electrophoretic analysis of the products of a deformylation reaction in which free F-Met-[^{14}C]Phe served as substrate (Figure 8). The products of the reaction are Met-Phe and Phe. Degradation of the unblocked dipeptide is likely accomplished by the aminopeptidase activity known to contaminate the partially purified extract (*cf.* Figure 2). The results indicate that both nascent F-Met-peptidyl-tRNA and free *N*-formyl peptides can serve as substrates for the reaction.

Anionic Activation of Deformylase. Despite the striking instability of the enzyme, the initial rate of the deformylation reaction is readily enhanced by high concentrations of SO_4^{2-} or SO_3^{2-}. As shown in Figure 9, the rate of the reaction is tripled by raising the concentration of $(NH_4)_2SO_4$ from 0.17 to 1.7 M. The data in Ta-

TABLE III: Sulfate and Sulfite Activation of Deformylase.[a]

Addition	Ionic Strength	[^{14}C]Formate Released ($\mu\mu$moles/min)
None	0.5	23
$(NH_4)_2SO_4$	4.2	88
Na_2SO_4	5.0	93
Na_2SO_3	3.4	115
NH_4Cl	4.0	12
NaCl	4.0	21

[a] Components of the reaction mixture and conditions of incubation are as described in Materials and Methods. The ionic strength of each reaction mixture is indicated in the table. Partially purified enzyme (3.75 μg) was used in each reaction mixture.

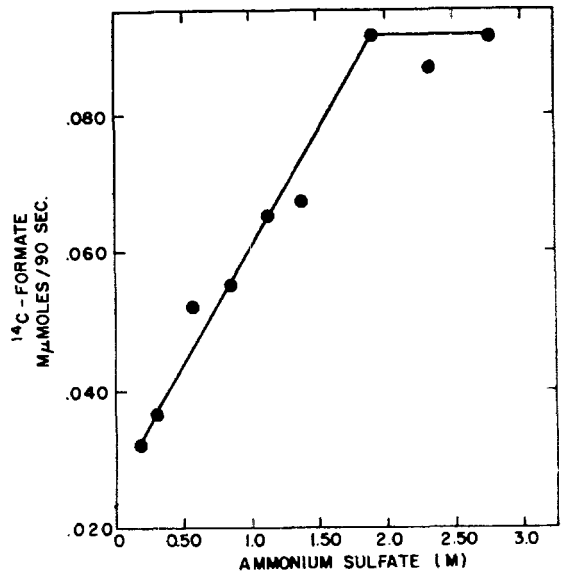

FIGURE 9: Effect of $(NH_4)_2SO_4$ concentration on the initial rate of deformylation reaction. Components of reaction mixtures are as described in Materials and Methods. In addition, each reaction mixture contained 3.75 μg of partially purified enzyme. Incubation was for 90 sec at 23°.

ble III indicate that the effect is not due to the accompanying cation or to ionic strength, but is relatively specific for SO_4^{2-} and SO_3^{2-}. Further studies carried out using salts of varied mono- and divalent cations and anions at both equimolar concentrations and/or equivalent ionic strength confirm the specificity of the SO_4^{2-}/SO_3^{2-} effect. Neither the related anions SeO_4^{2-} or PO_4^{3-} had any stimulatory effect on the activity. Although SO_4^{2-} increased the initial reaction rate, it was ineffective in protecting the enzyme from heat inactivation.

Discussion

The universal occurrence of F-Met in *E. coli* and RNA bacteriophage proteins synthesized in cell-free systems (Adams and Capecchi, 1966; Capecchi, 1966; Gussin *et al.*, 1966; Webster *et al.*, 1966) and the frequent absence of both blocking group and N-terminal methionine in these proteins when synthesized *in vivo* (Waller, 1963; Konigsberg *et al.*, 1966) led several workers (Adams and Cappechi, 1966; Webster *et al.*, 1966) to predict an enzymatic mechanism for their removal during protein biosynthesis. Gussin *et al.* (1966), Fry and Lamborg (1967), and Adams (1968) have reported the first of these required activities acting at the level of the free formyl peptide in bacterial extracts. It is likely, therefore, that the formyl group, while required for certain initial events in protein synthesis is redundant in terms of the activity of the finished protein product.

The experiments which we report relate to the point in protein synthesis at which the formyl group becomes available for deformylation and to the unique specificity of the deformylase enzyme in this process. A consideration of the role of the formyl group in protein biosynthesis suggests that deformylation might occur following incorporation of F-Met into the initial peptidyl-tRNA intermediate, the blocking group apparently not being required for further elaboration of the polypeptide chain (Nakamoto and Kolakofsky, 1966). With these expectations, we have chemically synthesized [^{14}C]F-Met-puromycin, an analog of F-Met-aminoacyl-tRNA, and have shown that it serves as a conveniently assayed substrate for a highly specific deformylation reaction 3. Early studies of peptide bond formation suggested that this might be the case (Hershey and Thach, 1967; Leder and Nau, unpublished observations). Using this synthetic substrate we have prepared a deformylation enzyme from extracts of *E. coli* which has several properties implicating it in formate removal during protein biosynthesis. Although extremely labile, the enzyme has been sufficiently purified to indicate that it acts *only* after the incorporation of the blocked amino acid into the initial peptidyl-tRNA intermediate. In contrast to other *E. coli* enzymes, this activity does not affect free F-Met (Birnbaum *et al.*, 1952; Vogel, 1953; Ohmura and Hayaishi, 1957; Fry and Lamborg, 1967; Weissbach and Redfield, 1967). Although these data provide further support for the redundancy of the formyl-blocking group during the propagative phase of protein assembly, other functions are not excluded. For example, α-amino-blocking groups provide efficient protection against aminopeptidase degradation (Smith and Spackman, 1955) and may protect nascent protein during synthesis (Bretscher and Marcker, 1966). It is clear from these studies that extracts of *E. coli* also contain enzymes which will remove N-terminal methionine from nascent peptides once deformylation has occurred. The specificity of this reaction has not been determined. It might represent the methionine-aminopeptidase reaction required to produce the mature form of bacterial and RNA bacteriophage proteins. Previous studies, however, indicate the presence of relatively nonspecific ribosomal and soluble aminopeptidase activities in bacterial extracts (Bolton *et al.*, 1959; Matheson, 1963; McCorquodale, 1963; Matheson and Tsai, 1965; Tsai and Matheson, 1965;

Matheson and Murayama, 1966; Simmonds and Toye, 1967). It is also possible that the presence or absence of the formyl group and/or N-terminal methionine affects the function of these proteins. The state of the N-terminal residue of *in vitro* synthesized proteins might be, in part, responsible for the difficulty in achieving cell-free synthesis of active enzymes. Recent evidence suggests, however, that removal of the formyl group is not required for release of peptide from tRNA during termination (Capecchi, 1967; Caskey *et al.*, 1968).[3]

Any detailed study of the specificity and kinetics of the deformylation reaction is complicated by the marked lability of the enzyme. Our own observations confirm those of Adams (1968) in this respect and indicate that differences in N-terminal residues observed between *in vivo* and *in vitro* synthesized proteins are likely accounted for by the rapid inactivation of deformylase in cell-free extracts. Another unusual feature of the enzyme is the effect of high concentrations of SO_4^{2-} and SO_3^{2-} on initial reaction rates. Though anionic enzyme activation is not uncommon, it generally occurs at relatively lower concentrations of anion and its specificity often extends to related anions (Barnard and Stein, 1959; Hirs, 1962; Cooke *et al.*, 1963; Crestfield *et al.*, 1963; Ginsburg and Carroll, 1965; Anderson and Nordlie, 1968). Such an effect often leads to stabilization of enzymatic activity by effects on enzyme conformation (Sela *et al.*, 1957; Nelson *et al.*, 1962; Neumann *et al.*, 1962; Craig *et al.*, 1963; Ginsburg and Carroll, 1965). Unfortunately, these anions do not protect deformylase from heat inactivation under the conditions we have studied. The possible involvement of a labile cofactor, though not entirely excluded, seems unlikely in view of our own attempts at cofactor replacement and the studies of Adams (1968).

Our studies raise the possibility that the formyl group is removed from nascent peptidyl-tRNA soon or immediately after formation of the initial peptide bond during protein synthesis. Removal of this group apparently makes N-terminal methionine available for *E. coli* aminopeptidase degradation, a reaction frequently required to produce the mature form of certain bacterial and viral proteins. An important question remains as to the role that the terminal blocking group and redundant amino acid might play in the functional activation of these proteins. Stabilization of reasonably specific preparations of the deformylase enzyme coupled with the activation effect of SO_3^{2-} and SO_4^{2-} should permit a direct approach to this question.

Acknowledgments

We are grateful to Dr. Meir Wilchek for his patient and expert advice on matters pertaining to chemical syntheses, to Mr. David Rogerson and his staff for preparation of the bacteria used in these studies, and to Mrs. Elizabeth Stotler for aiding us so greatly in the preparation of this manuscript.

[3] NOTE ADDED IN PROOF. Takeda and Webster (1968) have recently shown F-Met-Puro to be deformylated by extracts of *B. subtilis*.

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The Properties of Thyroglobulin. XVIII. Isolation of Thyroglobulin Subunits*

Peter Nissley,† N. Cittanova,‡ and H. Edelhoch

ABSTRACT: After mild reduction of bovine thyroglobulin with dithiothreitol at alkaline pH, followed by alkylation, two new, slower sedimenting species are observed by centrifugation. These species have been isolated in purified form by sucrose gradient centrifugation and found to have one-fourth to one-half the molecular weight of the native protein. Cleavage with cyanogen bromide of the isolated subunits, after extensive reduction and alkylation, revealed no differences by disc electrophoresis between these two species and native thyroglobulin. The number of disc electrophoretic bands observed was close to half the number of methionine residues present in thyroglobulin and implies that the different chains of thyroglobulin must exist in identical pairs.

The slowest sedimenting component therefore appears to be a mixture of two different subunits with very similar molecular properties. The faster sedimenting component(s) appears to be an unfolded form of the 12S dissociation product of thyroglobulin.

The effects of pH, urea, and mercaptoethanol on the extent of disulfide-bond reduction of 19S thyroglobulin have been reported recently (de Crombrugghe *et al.*, 1966). The molecular properties of two, new, slower sedimenting components observed at alkaline pH in dilute urea were evaluated by sedimentation velocity and viscosity. From these measurements it was suggested that the slower component was about one-half the size of 12 S and that the faster one was probably a dimer formed by association of the slower one. It has been shown that the 12S species is a subunit of 19 S observable under a variety of conditions where covalent bonds are unlikely to be broken, *i.e.*, low ionic strength, alkaline pH, in detergent, in urea, and in guanidine solution (Edelhoch, 1960; Edelhoch and Lippoldt, 1960, 1964).

It is the purpose of this communication to describe the isolation of the slower sedimenting subunit, to characterize some of its physical properties, and to compare its chemical properties with those of the native protein.

Materials and Methods

Preparation of Partially Reduced and Alkylated Thyro-

* From the Clinical Endocrinology Branch, National Institute of Arthritis and Metabolic Diseases, National Institutes of Health, Bethesda, Maryland 20014. *Received September 6, 1968.*

† Present address: Johns Hopkins School of Medicine, Baltimore, Md.

‡ Present address: Faculte de Medecine, Paris 6, France.