Inhibition of *N*-Acetylphenylalanyl Transfer Ribonucleic Acid Binding to 30S Ribosomal Subunit of *Escherichia coli* by *N*-Formylmethionyl Transfer Ribonucleic Acid†

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ABSTRACT: The poly(uridylic acid)-directed binding of AcPhe-tRNA to *Escherichia coli* 30S ribosomal subunits can be made pseudo first order in 30S subunit concentration with a rate constant of about 0.2 sec^{-1} and a half-life period of about 3 sec. The reaction is strongly stimulated by IF-3 in the presence of IF-1 and IF-2. Addition of fMet-tRNA to this model system markedly inhibits AcPhe-tRNA binding. The inhibition is by fMet-tRNA specifically; neither Met-tRNA$_f$ nor any other charged or uncharged tRNA inhibits, even at relatively high concentrations. The inhibition depends on IF-1 and IF-2, but not on IF-3. Analysis of the time course of AcPhe-tRNA binding in the presence of fMet-tRNA indicated that about half of the ribosomes are strongly inhibited by fMet-tRNA; the other half still bind AcPhe-tRNA rapidly, although with a slightly reduced rate constant of about 0.15 sec^{-1}. Differential sensitivity of the 30S ribosomal subunits to fMet-tRNA is also indicated by the observation that 50% inhibition is readily obtained with low concentrations of fMet-tRNA, while far higher concentrations are required for further inhibition. These results suggest that a significant fraction of the 30S ribosomal subunits is capable of interacting strongly with fMet-tRNA even in the absence of an appropriate mRNA.

The orthodox view of initiation of protein synthesis in bacteria postulates binding of the 30S ribosomal subunit to mRNA, followed by binding of fMet-tRNA. Binding of the ribosome to the messenger may be either specific or nonspecific (Nakamoto and Kolakofsky, 1966). With specific binding, the 30S ribosomal subunit (30S SU) itself recognizes and selects the initiator site of the mRNA; initiation factor IF-3 is held to mediate the binding (Iwasaki *et al.*, 1968; Revel *et al.*, 1969).

$$\text{30S SU} \xrightarrow[\text{(selection)}]{\text{mRNA}} \text{30S SU-mRNA} \xrightarrow{\text{fMet-tRNA}} \text{initiation complex} \quad (1)$$

With nonspecific binding of the ribosome, the initiator site is selected when binding of the ribosome attached to the proper site of the mRNA is stabilized by the binding of initiator tRNA (Nakamoto and Kolakofsky, 1966).

$$\text{30S SU} \overset{\text{mRNA}}{\longleftrightarrow} \text{30S SU}\cdots\text{mRNA} \xrightarrow[\text{(selection)}]{\text{fMet-tRNA}} \text{initiation complex} \quad (2)$$

Recently, evidence for a different pathway, in which the initiator tRNA is bound to the ribosome before mRNA, has been obtained in a eukaryotic system by the isolation of a messengerless complex of the 40S ribosomal subunit with Met-tRNA$_f$ (Baglioni, 1972; Darnbrough *et al.*, 1973; Schreier and Staehelin, 1973). Although no such evidence has been adduced for the bacterial systems, the analogous formulation would be

$$\text{30S SU} \overset{\text{fMet-tRNA}}{\longleftrightarrow} \text{30S SU-fMet-tRNA} \xrightarrow[\text{(selection)}]{\text{mRNA}} \text{initiation complex} \quad (3)$$

Our observation that initiation factor IF-3 strongly stimulates the binding of AcPhe-tRNA to the 30S ribosomal subunit of *E. coli* in the presence of initiation factors IF-1 and IF-2 (Bernal *et al.*, 1974) has encouraged us to use this model system to explore the alternative pathways for initiation of protein synthesis. If formation of a messengerless complex is the normal first step, as in pathway 3 above, then addition of fMet-tRNA to the model system could inhibit AcPhe-tRNA binding by formation of such a complex. If binding of initiator tRNA always follows binding of mRNA, as in pathways 1 and 2, no such inhibition would be expected. We have, therefore, undertaken a kinetic study of the effect of fMet-tRNA on the poly(U)[1]-directed binding of AcPhe-tRNA to the 30S ribosomal subunit.

Experimental Section

Materials. Quarter-log *E. coli* B cells were purchased from Grain Processing Co.; mid-log *E. coli* Q13 cells from General Biochemicals; poly(U) from Miles Laboratories; dextran sulfate (MW 5 × 10^5) from Pharmacia; *N*-5-formyltetrahydrofolate (Calcium Leucovorin Injection) from Lederle; and crude *E. coli* B tRNA from Schwartz Bioresearch. Purified tRNA^{fMet} from *E. coli* K12 MO was the generous gift of Dr. G. D. Novelli.

Methods. The preparation of Ac[^{14}C]Phe-tRNA, 1 M NH_4Cl washed ribosomes and subunits, and protamine-treated supernatant was carried out as described previously (Hamel *et al.*, 1972). In the preparation of f[^{3}H]Met-tRNA, *N*-5-formyltetrahydrofolate (Calcium Leucovorin Injection) was used as formyl donor (Nakamoto and Kolakofsky, 1966). The f[^{3}H]Met-tRNA was isolated by adsorption onto DEAE-cellulose equilibrated with a solution containing 20 mM imidazole-HCl (pH 7.0), 0.2 M NH_4Cl, and 10 mM $MgCl_2$. Elution was carried out by increasing the NH_4Cl concentration to 0.75 M, and the tRNA was concentrated by precipitation with 2 volumes of ethanol.

Initiation factors were purified from *E. coli* Q13 by a modification of the method of Hershey *et al.* (1971). Cells were ground with alumina and ribosomes isolated by centrifuging.

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[1] Abbreviation used is: poly(U), poly(uridylic acid).

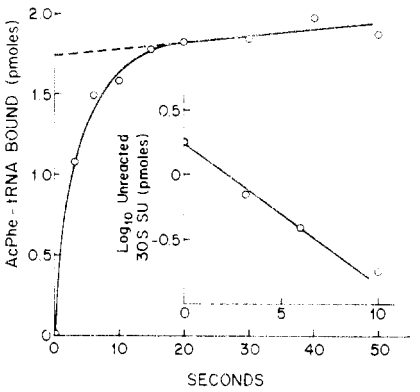

FIGURE 1: Kinetics of AcPhe-tRNA binding to 30S ribosomal subunits. Assay conditions were as described in Methods except that 6 μg of poly(U) was added. The dashed line, extrapolated to zero time, corrects for the 30S subunits which bind AcPhe-tRNA slowly. Inset: Plot of log of unreacted 30S subunits which bind AcPhe-tRNA rapidly *vs.* time.

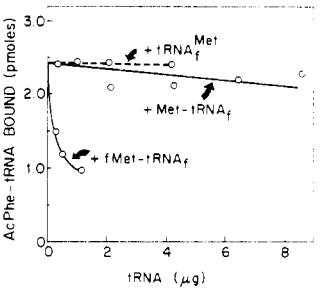

FIGURE 2: Specificity of inhibition of AcPhe-tRNA binding by fMet-tRNA. Assay conditions were as described in Methods. Where indicated, f[^{3}H]Met-tRNA containing 25 pmol of methionine per μg of RNA, [^{3}H]Met-tRNA containing 10 pmol of methionine per μg of RNA, and uncharged, purified $tRNA^{fMet}$ were added prior to the equilibration step. Incubation was for 10 sec.

The 30–85% $(NH_4)_2SO_4$ fraction of a 1.1 M NH_4Cl ribosomal extract obtained from 300 g of cell paste was passed rapidly through a 2 × 25 cm column of Sephadex G-25 equilibrated with a solution containing 20 mM imidazole-HCl (pH 7.4), 0.1 M NH_4Cl, 10 mM 2-mercaptoethanol, and 10% glycerol. Fractions in the void volume were run immediately onto a 1.5 × 20 cm column of phosphocellulose equilibrated with the same buffer. The column was washed with 600 ml of buffer, whereupon 1 l. of a 0.1–0.8 M NH_4Cl gradient was applied. Initiation factors were located by measuring the stimulation of AcPhe-tRNA binding to 30S ribosomal subunits in the presence of the other two factors, as described below. IF-2 activity appeared in the gradient between 0.25 and 0.35 M NH_4Cl, IF-1 between 0.35 and 0.45 M, and IF-3 between 0.50 and 0.65 M. The separated factors were concentrated on small phosphocellulose columns, then further purified by chromatography on Sephadex columns equilibrated with 20 mM Tris-HCl (pH 7.4), 0.4 M NH_4Cl, 10 mM 2-mercaptoethanol, and 10% glycerol. IF-1 eluted from a 1.5 × 80 cm column of G-50 (fine) at about two void volumes as a peak in fractions essentially devoid of 280-nm absorbance. IF-2 eluted from a 2 × 80 cm column of G-200 at 1.5 void volumes. IF-3 eluted from a 2 × 50 cm column of G-100 in a broad double peak commencing at 1.5 void volumes. The factors were again concentrated on small phosphocellulose columns, dialyzed briefly against 20 mM Tris-HCl (pH 7.4), 0.4 M NH_4Cl, 5 mM 2-mercaptoethanol, and stored at −70° where they are stable indefinitely. Plastic columns and tubes were used throughout.

In the assay for AcPhe-tRNA binding to 30S subunits, the complete reaction mixture contained in a final volume of 0.1 ml: 50 mM imidazole-HCl (pH 7.4), 8 mM $MgCl_2$, 80 mM NH_4Cl, 4 mM 2-mercaptoethanol, 0.2 mM GTP, 8 pmol of 1 M NH_4Cl-washed 30S ribosomal subunits preincubated 20 min at 37° in a medium containing 0.5 M NH_4Cl and 10 mM $MgCl_2$ (Nakamoto and Hamel, 1968; Zamir *et al.*, 1969), and sufficient initiation factors to give maximal rate of binding. About 0.2 μg of IF-1, 1.0 μg of IF-2, and 0.5 μg of IF-3 were generally more than sufficient. The mixture was equilibrated at 15°, and binding was initiated by adding 24 pmol of Ac[^{14}C]Phe-tRNA and 2 μg of poly(U). The reaction was stopped by rapid addition of 2.5 ml of ice-cold "binding buffer" containing 10 mM imidazole-HCl (pH 7.4), 8 mM $MgCl_2$, 80 mM NH_4Cl, and 5×10^{-6} M dextran sulfate. Dextran sulfate was dialyzed at about 0.1 mM concentration against 0.1 M NH_4Cl to eliminate free sulfate and phosphate ions. The ribosomal complexes were then adsorbed onto Millipore filters, washed twice with 3 ml of binding buffer, dried, and counted in a liquid scintillation system. The efficiency of counting was about 89% for ^{14}C and 40% for ^{3}H.

Results

The Basic System. A typical time course of the poly(U)-directed, Ac[^{14}C]Phe-tRNA binding to isolated 30S ribosomal subunits is shown in Figure 1. An initially rapid binding is essentially complete within 20 sec, and at this point a slow binding occurring at a linear rate becomes evident. We eliminate this slow reaction from consideration by extrapolating the curve back to zero time (dashed line), and evaluating the amount of fast-binding 30S subunits unreacted at any time by the difference between the extrapolated line and the experimental curve. The fast reaction then appears to be homogeneous and pseudo first order in 30S subunit concentration. The half-life period of the fast reaction is about 3.0 sec, and a rate constant of $k \simeq 0.25$ sec^{-1} can be calculated from the slope of the inset log plot which is equal to $-k/2.303$. Note that although 8 pmol of 30S ribosomal subunits was added, only 2 pmol of AcPhe-tRNA was bound.

Inhibition by fMet-tRNA. Figure 2 shows that small amounts of purified fMet-tRNA added to the binding system reduced the amount of AcPhe-tRNA bound in 10 sec by about 50%, whereas $Met\text{-}tRNA_f$ and $tRNA^{fMet}$, even in larger amounts, failed to inhibit this binding. To obtain further confirmation of this result, we devised a system wherein fMet-tRNA could be generated *in situ* from either purified $tRNA^{fMet}$ or from crude tRNA at a rate sufficiently slow that a preincubation step was required. Table I shows that the inhibition of AcPhe-tRNA binding depends on charging of tRNA with methionine specifically, and on formylation.

Effect of Initiation Factor Concentration on Inhibition by fMet-tRNA. Figure 3 shows that the degree of inhibition of AcPhe-tRNA binding was essentially invariant with initiation factor concentration, over a range from subsaturating to twofold excess. The initiation factor added in this experiment was a mixture of the three purified factors. The same result was obtained when purified initiation factors were titered individually in the presence of excess amounts of the other two factors (data not presented). This rules out the possibility that fMet-tRNA might act by sequestering one or more initiation factors, making them unavailable for AcPhe-tRNA binding, and thus giving only an apparent inhibition.

Inhibition as a Function of fMet-tRNA Concentration. The observed degree of inhibition of AcPhe-tRNA binding by fMet-tRNA was in the range of 50–70% when the binding reaction was carried out for periods of 10 sec or less. Generally,

TABLE I: Inhibition of AcPhe-tRNA Binding by fMet-tRNA Generated *in situ*.[a]

Conditions for fMet-tRNA	AcPhe-tRNA Bound (pmoles)	
	+ purified $tRNA^{fMet}$	+ crude tRNA
1. Complete	0.9	0.7
2. − Methionine	1.6	
3. − Methionine, + all other amino acids	1.6	1.6
4. − *N*-5-Formyltetrahydrofolate	1.8	1.6
5. − Supernatant enzymes	1.7	1.7
6. − tRNA	1.5	1.8

[a] Two mixtures were prepared containing in common 50 mM imidazole-HCl (pH 7.4), 8 mM $MgCl_2$, 80 mM NH_4Cl, 5 mM 2-mercaptoethanol, and 0.2 mM GTP. One mixture, maintained at 15°, contained in addition 8 pmol of 30S ribosomal subunits and 0.2 μg of IF-1, 1.0 μg of IF-2, and 0.5 μg of IF-3 in each 0.1-ml volume. The second mixture, distributed in 0.1-ml aliquots, contained 1 mM ATP, 2 mM phosphoenolpyruvate, and 0.1 μg of pyruvate kinase, and, where indicated, 20 mM methionine or all other amino acids, 10 mM *N*-5-formyltetrahydrofolate, 2 μl of protamine-treated supernatant and 0.8 μg of purified $tRNA^{fMet}$ or 25 μg of crude *E. coli* B tRNA. This mixture was preincubated 10 min at 37° and then transferred to the 15° bath where it was combined with 0.1 ml of the first mixture. Binding was initiated by adding 24 pmol of Ac[^{14}C]Phe-tRNA and 2 μg of poly(U), and was stopped after 10 sec with 3.5 ml of ice-cold buffer as described in Methods. Components omitted during the preincubation at 37° were added to the combined mixture just before AcPhe-tRNA and poly(U).

inhibition up to 50% was readily obtained by adding a few picomoles of fMet-tRNA in amounts approximately equimolar with 30S ribosomal subunits. Thereafter, the increment of inhibition decreased with increasing amounts of fMet-tRNA. This is somewhat apparent in Figure 2, and is obvious in Figure 4 where the first 10 pmol of fMet-tRNA accounts for an inhibition of nearly 40%, while 90 additional pmol of fMet-tRNA is required to increase the inhibition to 80%.

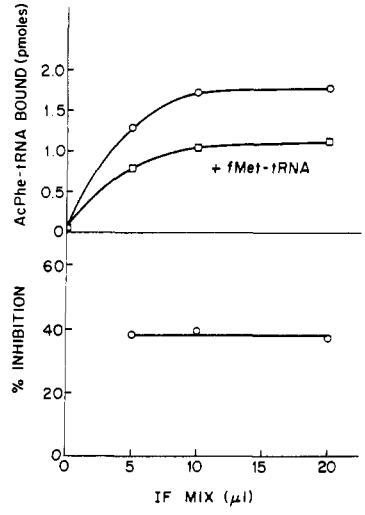

FIGURE 3: Inhibition of AcPhe-tRNA binding by fMet-tRNA with varied amounts of initiation factors. Assay conditions were as described in Methods. Purified initiation factors were mixed proportionately so that 10 μl contained saturating amounts of all three factors.

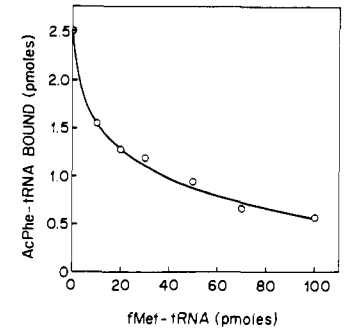

FIGURE 4: Inhibition of AcPhe-tRNA binding by increasing amounts of fMet-tRNA. Assay conditions were as described in Methods, with binding carried out for 6 sec; 1 μg of f[^{3}H]Met-tRNA preparation contained 8.5 pmol of methionine.

Kinetics of Inhibition of AcPhe-tRNA Binding by fMet-tRNA. Figure 5 shows the time course of AcPhe-tRNA binding with and without added fMet-tRNA. Extrapolations to correct for the slow binding are made as in Figure 1. In the absence of fMet-tRNA, the rapid binding of AcPhe-tRNA is complete by 20 sec, the half-life period is about 3.5 sec, and the inset log plot gives a value of $k \simeq 0.21$ sec^{-1}. As noted previously, the fast reaction is apparently homogeneous and pseudo first order in 30S subunit concentration. In the presence of fMet-tRNA, a rapid binding of AcPhe-tRNA can still be observed, but the rate is reduced slightly and the amount of rapid binding is only about 50% of that seen in the absence of fMet-tRNA. Moreover, after the rapid binding is completed, binding continues at a slow, linear rate which is somewhat faster than the slow binding observed in the absence of fMet-tRNA. The fast reaction again appears to be homogeneous and pseudo first order in 30S subunit concentration with a half-life period of about 5 sec and a rate constant $k \simeq 0.13$ sec^{-1}. These results, and those of Figure 3, suggest that there may be two major species of 30S ribosomal subunits which bind AcPhe-tRNA homogeneously in the absence of fMet-tRNA, but can be distinguished by their very different responses to fMet-tRNA. One species interacts relatively weakly with fMet-tRNA so that it still binds AcPhe-tRNA rapidly, while the other species interacts so strongly with fMet-tRNA that AcPhe-tRNA binding is almost completely inhibited. The increase in rate of the slow, linear binding of AcPhe-tRNA seen with added fMet-

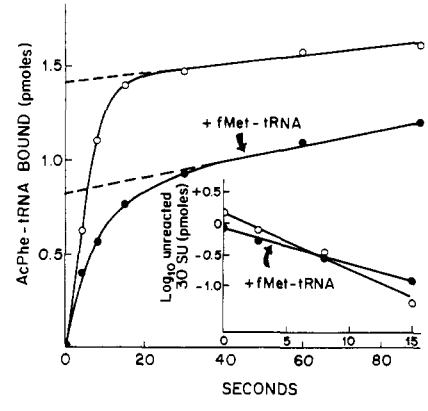

FIGURE 5: Effect of fMet-tRNA on the kinetics of AcPhe-tRNA binding to 30S ribosomal subunits; 0.5 μg of f[^{3}H]Met-tRNA containing 13 pmol of methionine was added where indicated. As in Figure 1, dashed extrapolations correct for 30S subunits that bind AcPhe-tRNA slowly. Inset: Plot of log of unreacted 30S subunits which bind AcPhe-tRNA rapidly *vs.* time.

TABLE II: Rate Constants for the Rapid Binding of AcPhe-tRNA in the Presence and Absence of fMet-tRNA.[a]

Expt	k (sec^{-1})		Ratio
	− fMet-tRNA	+ fMet-tRNA	
1	0.21	0.13	1.6
2	0.24	0.15	1.6
3	0.26	0.14	1.8

[a] Assay conditions were as described for Figure 5. The rate constants were evaluated from plots of log of unreacted 30S ribosomal subunits *vs.* time.

tRNA probably reflects the net rate of dissociation of fMet-tRNA from the strongly interacting 30S subunits.

Kinetic constants for the rapid binding in the presence and absence of fMet-tRNA derived from three separate experiments, including that shown in Figure 5, are summarized in Table II. If it is valid to assume a rapid equilibration between fMet-tRNA and the weakly interacting 30S subunits, then the results indicate that about 30% of these 30S subunits are complexed with fMet-tRNA, since the ratio of the rate costants $k(-\text{fMet-tRNA})/k(+\text{fMet-tRNA})$ is equal to $[1 + (f/k_D)]$ where f is the concentration of fMet-tRNA and K_D is the dissociation constant for the weak interaction. For these experiments, the value of K_D is about 1×10^{-7} M. The assumption of rapid equilibration is not unreasonable, since inhibition is enhanced only 5–10% when fMet-tRNA is preincubated with ribosomes in the reaction mixture before binding is initiated by the addition of AcPhe-tRNA and poly(U).

Effect of Preincubation with Poly(U) on Inhibition by fMet-tRNA. Poly(U) is known to bind relatively strongly to the 30S ribosomal subunit (Takanami and Okamoto, 1963), at the mRNA binding site (Van Duin and Kurland, 1970). We anticipated, therefore, that preincubating poly(U) with the ribosomes in the reaction mixture would hinder the interaction of fMet-tRNA, thus reversing its inhibition of AcPhe-tRNA binding. Experiments performed to test this prediction clearly showed, however, that added fMet-tRNA inhibited AcPhe-tRNA binding just as much when poly(U) was preincubated with the ribosomes as when the poly(U) was added to initiate binding (data not presented).

Effect of Individual Initiation Factors on the Inhibition of AcPhe-tRNA Binding by fMet-tRNA. Figure 6 shows the time course of AcPhe-tRNA binding with and without added fMet-tRNA, where IF-1, IF-2, and IF-3 are successively omitted. It is clear that the binding is strongly stimulated by all three initiation factors. Omission of IF-2 results in loss of binding, as well as of inhibition. The small amount of binding seen without IF-2 may be attributed in part to contamination of the IF-3 with some IF-2. Omission of IF-1 results in a reduced rate of binding and in a diminution of inhibition by added fMet-tRNA. Omission of IF-3 also results in a reduced rate of binding but, if anything, enhances the inhibition of AcPhe-tRNA binding by fMet-tRNA. It is of interest that the two initiation factors IF-1 and IF-2, which have been implicated in mediating the binding of initiator tRNA to the ribosome (Salas *et al.*, 1967; Hershey *et al.*, 1969), are required for the inhibition of AcPhe-tRNA binding to 30S ribosomal subunits by fMet-tRNA.

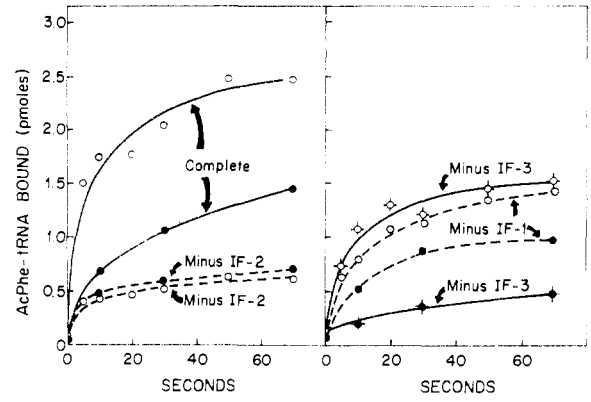

FIGURE 6: Effect of individual initiation factors on the inhibition of AcPhe-tRNA binding by fMet-tRNA. Assay conditions were as described for Figure 1; (○) AcPhe-tRNA binding in the absence of fMet-tRNA; (●) binding in the presence of fMet-tRNA. Where indicated, 0.2 μg of IF-1, 1.0 μg of IF-2, and 0.5 μg of IF-3, and 1.1 μg of f[^{3}H]Met-tRNA containing 20 pmol of methionine per μg of RNA were added.

Discussion

The present study shows that the poly(U)-directed binding of AcPhe-tRNA to *E. coli* 30S ribosomal subunits is inhibited specifically by fMet-tRNA; Met-tRNA$_f$, tRNA^{fMet}, and all other tRNAs, whether aminoacylated or not, are ineffective. Initiation factor IF-2 appears to be critical for inhibition by fMet-tRNA; IF-1 enhances the inhibition in the presence of IF-2, whereas IF-3 is not essential. Since fMet-tRNA does not inhibit the binding of AcPhe-tRNA by sequestering initiation factors, as evidenced by the constancy of inhibition with increasing amounts of the factors, nor by sequestering GTP which was always in excess, fMet-tRNA probably inhibits by competing for a site on the 30S ribosomal subunit. The requirement for inhibition of IF-1 and IF-2 which have been implicated in mediating the interaction of fMet-tRNA with the 30S subunit also supports this conclusion.

The 30S ribosomal subunits appear to be heterogeneous in their interaction with fMet-tRNA, even though they behave homogeneously in binding AcPhe-tRNA in the absence of fMet-tRNA. Heterogeneity was indicated by the 50% inhibition of AcPhe-tRNA binding obtained readily at concentrations of fMet-tRNA about equimolar with the 30S ribosomal subunits, and by the smaller incremental inhibition beyond 50% obtained only with much larger amounts of fMet-tRNA. Heterogeneity was also indicated by the time course of AcPhe-tRNA binding in the presence of fMet-tRNA: about one-half of the 30S subunits still bound AcPhe-tRNA rapidly, with only a slight reduction in rate, while the other half were almost completely inhibited from binding AcPhe-tRNA. Perhaps of relevance here is the heterogeneity of 30S ribosomal subunits to *N*-ethylmaleimide treatment; under certain experimental conditions only one-half of them were inactivated (Moore, 1973; Ginzburg *et al.*, 1973). Possibly, the isolation of more proteins from 30S ribosomal subunits than are contained in any one subunit is also relevant (Craven *et al.*, 1969).

Although the possibility of AcPhe-tRNA binding occurring on two different ribosomal sites has not been rigorously excluded, this is not considered too likely since essentially all of the AcPhe-tRNA bound to the 30S subunit reacts with puromycin when 50S ribosomal subunits are added in the absence of elongation factor G (unpublished observations). Moreover, it would be unusual for the rate of AcPhe-tRNA binding to two different ribosomal sites to be identical, especially when the binding is catalyzed by the same three initiation factors that catalyze the binding of fMet-tRNA to the 30S ribosomal subunit presumably at a specific site.

Finally, the observation that fMet-tRNA exerts its inhibitory effect not only in the absence of a mRNA containing the appropriate AUG or GUG codons but in the presence of poly(U), which should interfere with the interaction of fMet-tRNA and the 30S ribosomal subunit, indicates that fMet-tRNA has strong affinity for the 30S subunit. Thus, even though the formation of a messengerless complex of the 30S subunit and fMet-tRNA has not been demonstrated directly, the participation of such a complex in the initiation of bacterial protein synthesis as in pathway 3 must be considered a possibility, especially with the 30S ribosomal subunits that are strongly inhibited by fMet-tRNA binding. On the other hand, the 30S subunits which interact weakly with fMet-tRNA, if they are not damaged ribosomes, may initiate protein synthesis by either pathway 2 or pathway 3, since other suggestive evidence obtained in this laboratory (Bernal *et al.*, 1974) rules out pathway 1.

Acknowledgment

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Nonequivalence of Chains in Hemoglobin Oxidation and Oxygen Binding. Effect of Organic Phosphates†

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ABSTRACT: Oxidation of Hb A is governed by at least two factors: (a) the intrinsic susceptibility to oxidation of chains which depend on their primary structure, and (b) the affinity of chains for oxygen or other ligands. 2,3-Diphosphoglycerate (2,3-DPG) stabilizes the deoxy conformation of hemoglobin which has a lower affinity for oxygen. This effect is maximal at acid and minimal at alkaline pH. Hemoglobin F_1 which does not bind organic phosphates does not show significant affinity changes in their presence (H. F. Bunn and R. W. Briehl (1970), *J. Clin. Invest. 49,* 1088). The present paper shows that: (a) the presence of 2,3-DPG in hemoglobin solutions increases the rate of autoxidation at acid pH where the binding is the most significant; this effect is correlated with the decrease in affinity for oxygen; (b) although the organic phosphates bind to non α chains, their effect is mostly exerted on the oxygen affinity of α chains.

The autoxidation rate of the four heme groups of hemoglobin depends on at least two factors: (1) the primary structure and its consequences on the chain concerned, and (2) the presence and type of ligand (Mansouri and Winterhalter, 1973).

Organic phosphates, notably 2,3-diphosphoglycerate (2,3-DPG),[1] are present inside the red cell in a concentration approximately equimolar to that of hemoglobin. The level of 2,3-DPG is an important regulator of oxygen affinity of the blood (Benesch *et al.*, 1968; Benesch and Benesch, 1969; Chanutin and Curnish, 1967; Rörth, 1968; Lo and Schimmel, 1969; Waldeck and Zander, 1969). It has been shown that in hypoxic states the level of 2,3-DPG increases with a concomitant decrease in oxygen affinity (Lenfant *et al.*, 1968; Eaton *et al.*, 1969).

Lowering the oxygen affinity of hemoglobin is achieved by a shift of the allosteric equilibrium in favor of the T (deoxy) con-

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[1] Abbreviation used is: 2,3-DPG, 2,3-diphosphoglycerate.