Stryer, L. (1965), *J. Mol. Biol. 13,* 482.

Thompson, W., and Yielding, K. L. (1968), *Arch. Biochem. Biophys. 126,* 399.

Warren, S. G.. Edwards, B. F. P., Evans, D. R., Wiley, D. C., and Lipscomb, W. N. (1973), *Proc. Nat. Acad. Sci. U. S. 70,* 1117.

Weber, G., and Young, L. B. (1964), *J. Biol. Chem. 239,* 1415.

Weissbach, H., Miller, D. L., and Hachmann, J. (1970), *Arch. Biochem. Biophys. 137,* 508.

Werber, M., Szent-Gyorgyi, A. G., and Fasman, G. D. (1972), *Biochemistry 11,* 2872.

Reversible Equilibrium in the Reaction between Ribosomes and the Dissociation Factor of *Escherichia coli*†

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ABSTRACT: Various features of the dissociation of free ribosomes of *Escherichia coli* by initiation factor IF$_3$ have suggested that the reaction is readily reversible. This reversibility is now demonstrated: ribosomes that have come to equilibrium with the factor rapidly shift their dissociation, to the expected value, when the concentration of the reactants is changed. A similar reequilibration is observed on raising or lowering the concentration of Mg^{2+}, which evidently influences the equilibrium constant. Moreover, when labeled ribosomes were added to an equilibrated mixture it could be shown that the first set of ribosomes partly reassociated and the second set dissociated, both reaching the same equilibrium value.

The ribosome dissociation factor (DF)[1] of *Escherichia coli* (Subramanian *et al.*, 1969), subsequently identified with initiation factor IF$_3$ (Sabol *et al.*, 1970; Subramanian and Davis, 1970), causes net dissociation of free ribosomes by forming a complex with the 30S subunit (Subramanian *et al.*, 1968; Parenti-Rosina *et al.*, 1969; Sabol and Ochoa, 1971). Since free ribosomes are normally in equilibrium with a low concentration of free subunits (Infante and Baierlein, 1971), the complexes can conceivably be formed either by interaction of DF with the free 30S subunits or by direct attack on the 70S ribosomes (Davis, 1971). But whichever the actual sequence, the overall reaction appears to involve a rapidly reversible, Mg^{2+}-dependent equilibrium rather than a stoichiometric titration, since the amount of dissociation by added IF$_3$ varies strikingly with Mg^{2+} concentration and is far less than a molar equivalent (Subramanian and Davis, 1970).

Such an equilibrium would be consistent with the observed rapid exchange of subunits between "heavy" and "light" labeled ribosomes mixed shortly after runoff (Subramanian and Davis, 1971), though it is not certain how much of this exchange involves interaction of the particles with DF. On the other hand, it has been reported that free ribosomes previously isolated from a sucrose gradient failed to exchange subunits with ribosomal particles being released from polysomes in their presence, and they also failed to compete effectively with these particles for dissociation ("anti-association") by a limited supply of DF (Kaempfer, 1970, 1971, 1973). Since the discrepancy has significant bearing on the ribosome cycle (Subramanian and Davis, 1973) it seemed important to test directly for the reversibility of the DF reaction.

In order to approximate physiological conditions we used a crude preparation of initiation factors as DF; key experiments were confirmed with partly purified or with pure IF$_3$. The results will show that freshly released ribosomes reach a reversible equilibrium with DF and subunits, and the subunits exchange rapidly with subsequently added differentially labeled ribosomes. Since this work was completed Sabol *et al.* (1973) have demonstrated reversibility with labeled IF$_3$ rather than labeled ribosomes: the IF$_3$ binds reversibly to free 30S subunits and does not bind to 70S ribosomes.

Materials and Methods

Bacterial Preparations. Strain MRE600 of *E. coli*, lacking RNase I, was grown in minimal medium A (Davis and Mingioli, 1950) supplemented with 0.2% glucose and 0.2% Casamino acids as previously described (Beller and Davis, 1971).

Runoff ribosomes were prepared by pelleting from lysates of slow-cooled cells (Beller and Davis, 1971); NH$_4$Cl-washed ribosomes were prepared according to the method of Iwasaki *et al.* (1968), with modifications, from an S30 extract in TNMD buffer (10 mM Tris-HCl (pH 7.8), 60 mM NH$_4$Cl, 10 mM Mg(Ac)$_2$, 2 mM dithiothreitol) with 0.5 μg/ml of DNase (Worthington). In this procedure the ribosomes were repelleted, dissolved in TNMD buffer + 20% glycerol to an A_{260} of 200–500, and stored at 0°.

Crude DF was prepared from the 1 M NH$_4$Cl supernatant (Iwasaki *et al.*, 1968) by slow addition of (NH$_4$)$_2$SO$_4$ to 70% saturation; the precipitate was stored at −70°. For use in DF assays part of the precipitate was dissolved, at 10–20 mg of protein/ml, in TKM$_5$D (10 mM Tris-HCl (pH 7.6), 50 mM KCl, 5 mM Mg(Ac)$_2$, 2 mM dithiothreitol), and was dialyzed against the same buffer for 1–2 hr. Partly purified IF$_3$ was

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[1] Abbreviations used are: DF, dissociation factor; IF, initiation factor(s); TKMD and TNMD, buffers described under Materials and Methods.

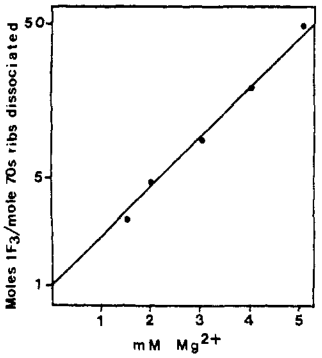

FIGURE 1: Stoichiometry of dissociation of 70S ribosomes by IF_3 at various Mg^{2+} concentrations. The data are from Subramanian and Davis (1970), Figure 3. They have been replotted to show the molar ratio of IF_3 to ribosomes dissociated at equilibrium, utilizing values of 2.1×10^4 (Sabol *et al.*, 1970) and 2.6×10^6 for the molecular weights of IF_3 and 70S ribosomes, respectively.

prepared through the phosphocellulose stage by the method of Sabol *et al.* (1970). Pure IF_3 was prepared essentially according to Dubnoff and Maitra (1971) with the 0–70% $(NH_4)_2SO_4$ precipitate as starting material.

Preparation of Radioactively Labeled Ribosomes and Subunits. For ^3H-labeled runoff ribosomes cells were grown for several generations in medium containing [^3H]uracil (0.8 μCi/0.5 μg per ml) (Schwarz) to a density of 5×10^8/ml (estimated from optical density). Unlabeled uracil (100 μg/ml) was added and growth was continued for 10 min to chase out labeled mRNA. The culture was cooled slowly to 15° for 20 min and the ribosomes, predominantly runoff, were pelleted without isolation and were stored as described above for NH_4Cl-washed ribosomes, except that the salt washing was omitted.

For ^{32}P-labeled gradient-isolated runoff ribosomes cells were grown at 37° to 6×10^8/ml in 25 ml of a medium containing 1% Bacto-peptone, 0.2% glucose, and 0.5% NaCl; the PO_4^{3-} content was 0.73 mM (method of Fiske and Subbarow, 1925). The culture was diluted 1:3 with Tris-A medium (medium A in which the PO_4^{3-} is replaced by 0.05 M Tris-HCl (pH 7.4)) containing 0.2% glucose, 0.1% Casamino acids, and 20 μCi/ml of carrier-free $H_3{}^{32}PO_4$ (New England Nuclear). Growth continued, without lag, until the culture reached a densiy of 4×10^8 cells/ml; 0.2 mM potassium phosphate (pH 7.0) was then added and growth resumed immediately. When the density reached 6×10^8 cells/ml the culture was cooled slowly to 15° for 20 min, and lysates were prepared and 70S runoff ribosomes were isolated from sucrose gradients as already described (Beller and Davis, 1971).

Results

Relation of DF Action to Mg^{2+} Concentration. Two possible mechanisms are readily visualized for a reversible dissociation reaction that yields a 30S·DF complex

$$70S + DF \rightleftharpoons [70S\cdot DF] \rightleftharpoons 30S\cdot DF + 50S \qquad (1)$$

or

$$70S \rightleftharpoons 30S + 50S \qquad (2a)$$

$$30S + DF \rightleftharpoons 30S\cdot DF \qquad (2b)$$

For either mechanism the equilibrium constant K, at constant Mg^{2+} concentration, is given by the relation

$$[30S\cdot DF][50S]/[70S][DF] = K \qquad (3)$$

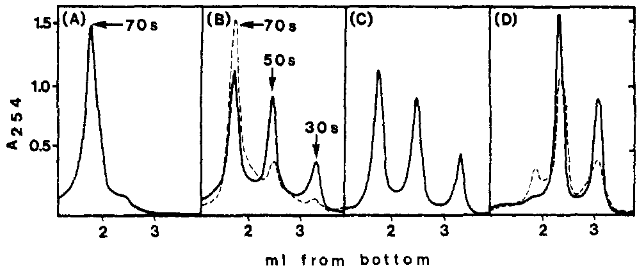

FIGURE 2: Reassociation by elevated Mg^{2+} of subunits formed by DF or by low Mg^{2+}: (A) initial runoff ribosomes; (B) DF-derived subunits: in 0.1 ml of TKM_5D buffer 30 μg of runoff 70S ribosomes was incubated for 15 min at 30° with 64 μg of crude DF (–); part was further incubated for 15 min with the Mg^{2+} raised to 15 mM (- - -); (C) reversal of effect of Mg^{2+}: as in B after exposure to 15 mM Mg^{2+}, but with an additional 15 min of incubation with the Mg^{2+} reduced again to 5 mM by dilution with TKD; (D) low Mg^{2+} subunits: as in B, except that initial incubation was with buffer containing 0.25 mM Mg^{2+} instead of with DF (–); part was further incubated with 15 mM Mg^{2+} as in (B) (- - -). After each incubation samples were layered on a chilled sucrose gradient and analyzed.

As noted above, the effectiveness of DF in causing net dissociation of free ribosomes of *E. coli* varies strikingly with Mg^{2+} concentration. Since the overall equilibrium is sensitive to Mg^{2+}, while the equilibrium between DF and derived 30S subunits is not (Zitomer and Flaks, 1972), the effect of Mg^{2+} appears to be exerted on reaction 2a.

A replot of the data of Subramanian and Davis (1970) reveals a linear relation between Mg^{2+} concentration and the logarithm of the molar ratio of DF added to ribosomes dissociated (Figure 1). The theoretical basis for this relation is not clear, but it is of interest that the DF/dissociation ratio, ranging from 50 to 2.5 in the range studied, extrapolates to a value of 1.0 at 0 Mg^{2+} concentration (which cannot be tested because of the spontaneous dissociation). This extrapolation, like the direct binding studies of Sabol *et al.* (1973), supports the conclusion that 1 molecule of DF binds to a 30S subunit.

Reversibility of the Effect of Mg^{2+} on DF-Mediated Dissociation. To test for the reversibility of the action of DF, ribosomes were incubated at 30° with a limiting amount of crude DF at 5 mM Mg^{2+}, and after the reaction had reached completion the Mg^{2+} concentration was elevated and incubation was continued for 15 min. After rapid chilling to fix the equilibrium (Subramanian *et al.*, 1968) a sample was analyzed in a sucrose gradient containing 5 mM Mg^{2+}. Samples fixed with 1% glutaraldehyde before transfer to the gradient (Subramanian and Davis, 1971; Subramanian, 1972) gave identical results, indicating the absence of reequilibration between the deposit of a sample on the chilled sucrose gradient and the start of centrifugation.

As Figure 2B shows, the subunits initially formed by DF were almost completely reassociated by exposure to 15 mM Mg^{2+} at 30°. Moreover, this exposure did not decrease the subsequent activity of either the DF or the ribosomes, for reduction of the Mg^{2+} concentration again to 5 mM restored the level of dissociation initially observed at that concentration (Figure 2C). These findings indicate that if the low efficiency of dissociation by DF involves adsorption of the factor to the glass (which has been observed during purification) that adsorption must be as reversible and Mg^{2+} dependent as the interaction with ribosomal particles.

Reequilibration on Sequential Addition of Ribosomes. The results presented strongly suggest a reversible equilibrium between ribosomes, DF, and subunits. More direct evidence was obtained by allowing labeled ribosomes to react with a

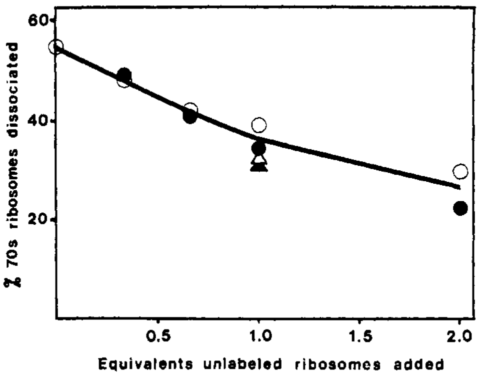

FIGURE 3: Effect of ribosome concentration on equilibration of undissociated ribosomes, subunits, and DF. *Sequential addition* (●,○): 90 μg of ^{3}H-labeled NH$_4$Cl-washed ribosomes (73% 70 S) was incubated for 15 min at 30° with 120 μg of crude DF in 0.6 ml of TKM$_3$D buffer. The reaction mixture was then divided into 0.1-ml portions (containing 15 μg of labeled ribosomes), different amounts of unlabeled NH$_4$Cl-washed ribosomes (also 73% 70 S) in 2 μl of TKM$_3$ were added as indicated, and incubation was continued for 15 min before analysis. *Simultaneous addition* (▲,△): 14 μg of labeled and 15 μg of unlabeled ribosomes were mixed in 0.1 ml of the same medium as above before adding 20 μg of DF and incubating for 30 min. Samples were layered on chilled sucrose gradients and analyzed for the distribution of total ribosomes (A_{254}) (●,▲) and radioactive ribosomes (○,△); % 70S dissociated = [% 70S initial (control) − % 70S final (sample)]/% 70S initial (control) × 100.

limiting amount of DF and then observing the effect of adding unlabeled ribosomes (with Mg^{2+} held at 5 mM).

As Figure 3 shows, after such an addition, increasing the total ribosome concentration 1.5- to 3-fold, the dissociated fraction of the total ribosomes decreased. Moreover, the initial set of ribosomal particles underwent partial reassociation and the added sample underwent partial dissociation, both reaching the same final level as when mixed (at the same total concentration) before incubation.

The redistribution was even more striking when the ratio of labeled to unlabeled ribosomes in such a two-stage experiment was decreased to 1:10. At such a low proportion the labeled ribosomes, added to unlabeled ribosomes already equilibrated with DF, reached the level of dissociation already attained by the latter ribosomes (Figure 4A). Conversely, when the dilute radioactive ribosomes were incubated first, with only one-twelfth as much DF (to yield 35% dissociation), addition of a tenfold excess of unlabeled ribosomes reduced the dissociation of the labeled ribosomes to an unmeasurable level (Figure 4B). Moreover, the half-time for this reassociation was about the same (3–4 min at 30°) as that for dissociation by DF, as would be expected for an equilibration.

The experiments of Figure 4 were performed with crude IF. Identical results were obtained with ribosomes dissociated by pure IF$_3$.

Contrast between Interaction with DF and with Mg^{2+}. The dissociation of ribosomes by DF, and its reversal, differ in kinetics and in sensitivity to temperature from the similar reactions induced by alterations in Mg^{2+} concentration. Thus, low Mg^{2+} induces complete dissociation within seconds (Zitomer and Flaks, 1972), in contrast to the half-time of 3–4 min for the DF reaction under the experimental conditions employed. Reassociation of DF-derived substrates was much faster when caused by elevated Mg^{2+} (unpublished) than when caused by redistribution of the DF (Figure 4). Moreover, DF caused little dissociation in 15 min at 0°, as previously observed (Subramanian *et al.*, 1968), while elevated Mg^{2+} reassociated DF-derived subunits almost completely after 2 min at 0°, just as at 37° (Table I). It thus appears that the reassociation of DF-derived subunits by elevated Mg^{2+} involves not simply displacement of the DF but conformational changes in the ribosome that accelerate the association reaction and that overcome the congealing effect of low temperature. These results are consistent with the known effect of Mg^{2+} on the spontaneous equilibrium between free ribosomes and their subunits at low temperature (Infante and Baierlein, 1971; Zitomer and Flaks, 1972).

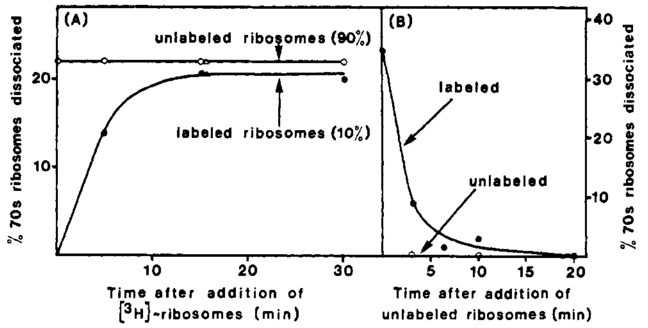

FIGURE 4: Kinetics of equilibration of added ribosomes with particles preincubated with DF. (A) Unlabeled NH$_4$Cl-washed ribosomes (300 μg) were incubated for 15 min at 30° with 275 μg of crude DF in 250 μg of TKM$_{5.6}$D buffer and a 50-μl sample (zero time) was then withdrawn and chilled. To the remainder 24 μg of ^{3}H-labeled runoff ribosomes (*i.e.*, 0.1 × the unlabeled ribosomes) was added and incubation was continued; 50-μl samples were withdrawn as indicated and analyzed for unlabeled (○) and labeled (●) ribosomes. Gradients were analyzed as in Figure 3, with the A_{254} tracings taken to measure the unlabeled ribosomes. A control mixture of unlabeled and labeled ribosomes and DF (in the same concentrations as above) was similarly incubated and then analyzed for unlabeled (△) and labeled (▲) ribosomes. (B) ^{3}H-Labeled runoff ribosomes (30 μg) were incubated with 22 μg of crude DF in 250 μl of buffer as in A, except that Mg^{2+} was 3.6 mM. After 15 min a 50-μl sample was withdrawn, chilled, and mixed with 60 μg of unlabeled ribosomes (zero-time sample). To the remainder 240 μg of unlabeled NH$_4$Cl-washed ribosomes (*i.e.*, 10 × the labeled ribosomes) was added, incubation was continued, and samples were withdrawn as in A.

Distortion of the Equilibrium by Damaged Particles. Earlier studies (Subramanian and Davis, 1970) showed that at 3–3.6

TABLE I: Ribosome Dissociation by DF and Its Reversal by Elevated Mg^{2+} at 0 and 30°.[a]

Tube	DF	Incubation I (3 mM Mg^{2+}, 15 min), Temp (°C)	Incubation II (13 mM Mg^{2+}, 2 min), Temp (°C)	% of Initial 70S Dissociated	% Reassn after Incubation II
a	+	0		14	
b	+	30		70	
c	+	30	0		82
d	+	30	30		88

[a] Incubation mixtures contained 15 μg of NH$_4$Cl-washed ribosomes (73% 70 S) and 20 μg of crude DF in 0.5 ml of TKM$_3$D buffer. After 15 min at the indicated temperature (incubation I), the mixtures were chilled in ice and the Mg^{2+} concentration of c and d was raised to 13 mM. After 2 min at the indicated temperature (incubation II), samples were layered on chilled sucrose gradients in TKM$_5$ and analyzed.

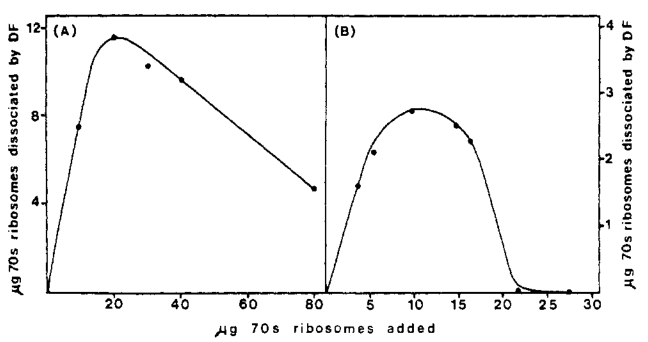

FIGURE 5: Inhibition of DF activity at high concentrations of NH_4Cl-washed ribosomes. Final incubation mixtures, in 0.2 ml of $TKM_{2.6}$ buffer, contained NH_4Cl-washed ribosomes (67% 70 S) as indicated and either (A) 34 µg of crude DF or (B) 5 µg of partially purified DF (see Methods). After 15 min at 30° samples were layered on chilled gradients and analyzed.

mM Mg^{2+} (as in Figures 3 and 4B), and at the ribosome concentrations usually tested, DF dissociates only about one-tenth its molar equivalent of ribosomes (Figure 1); hence, in the equilibrium of eq 3 the remaining nine-tenths of the DF molecules should be free. However, the results obtained on varying the ribosome concentration did not fit this equation well. For example, in the experiment of Figure 4B a severe reduction in the amount of ribosomes markedly reduced the amount of DF required for partial dissociation, which would not be expected if nine-tenths of the DF were free. Similarly, the subsequent tenfold increase in ribosome concentration reduced the dissociation from 35% to an undetectable level, rather than to the calculated (and easily detected) level of 15%. Indeed, when this unexpected observation was pursued further, extending the observations to even higher ribosome/DF ratios, the total dissociation by a fixed amount of DF was found to go through a maximum with an increase in ribosomes and then to decline. Moreover, this paradoxical effect was observed not only with crude IF but also with partly purified IF_3 (Figure 5).

Though these results seemed at first to conflict seriously with the proposed equations, the effect can now be seen to be an artefact, due to the use of NH_4Cl-washed ribosome preparations containing many damaged particles (including 25–30% nonassociable subunits). When runoff ribosomes were used instead, in a similar concentration range, the dissociation varied with ribosome concentration essentially as expected from eq 3 (Table II). (Indeed, the deviations in the equilibrium constant, presumably due to an analytical error, are opposite in direction to those expected from inhibition by excess ribosomes.) The contrast between the two preparations suggests that at least part of the particles in the NH_4Cl-washed preparations have suffered damage that prevents them from participating in the dissociation–association equilibrium but permits them to bind IF_3 with high affinity.

Discussion

In the reaction of free ribosomes of *E. coli* with DF the variation of the degree of dissociation with Mg^{2+} concentration originally suggested a reversible reaction of the form

$$30S \cdot 50S + DF \rightleftharpoons 30S \cdot DF + 50S$$

with a Mg^{2+}-dependent equilibrium constant (and with the possible participation of free 30S subunits as intermediates). This inference is now supported by several findings: (a) an increase or decrease in Mg^{2+} concentration in an already incubated mixture shifted the dissociation to the predicted value (Figure 2); (b) with labeled ribosomes preincubated with DF addition of unlabeled ribosomes caused net reassociation of the labeled subunits, and the two sets of ribosomes reached the same degree of dissociation (Figures 3 and 4); and (c) the rate of reassociation of labeled subunits after adding unlabeled ribosomes was essentially the same as the rate of net dissociation of ribosomes on adding DF (half-time 3–4 min at 30° and 3 mM Mg^{2+}). (These rates are much lower than the rates of the same reactions induced by marked changes in Mg^{2+} concentration.)

TABLE II: Increasing Total Dissociation with Increasing Concentration of Runoff Ribosomes.[a]

RS	% Dissociated	K'	SU
12.5	32	1.9	4.0
24.4	26	2.3	6.4
41	22	2.5	9.0

[a] RS, micrograms of 70S ribosomes present initially; SU, micrograms of subunits formed. Final incubation mixtures in 0.1 ml of TKM_3D buffer contained runoff ribosomes as indicated and 36 µg of crude DF (a different preparation from that of Figure 5). After 5 min at 37° samples were layered on chilled gradients and analyzed. The amount of ribosomes added, and the subunits measured, are corrected for the background content of subunits in each control mixture incubated without DF (30–34%). Assuming that the reservoir of free DF remained essentially constant, the equilibrium constant is calculated as: $[SU]^2/[RS] = K/[DF] = K'$.

Though increases in ribosome concentration shifted the ratio of subunits to ribosomes in the expected direction, the shift in net dissociation did not initially correspond to theory. In fact, an excess of ribosomes decreased the net dissociation by a given amount of DF, instead of shifting the equilibrium, as expected, in the opposite direction (Figure 5). However, this effect was found to be due to the use of NH_4Cl-washed ribosomes, which contained a substantial fraction of damaged subunits that could no longer reassociate but evidently competed for DF. With runoff ribosomes, which have been less extensively manipulated, the variation of dissociation with ribosome concentration was reasonably consistent with the postulated equilibrium (Table II).

Initially we had attempted to demonstrate the proposed equilibration more simply by isolating radioactively labeled DF-derived 30S and 50S subunits from a sucrose gradient and following the exchange of either with unlabeled free 70S ribosomes. However, we were unable to demonstrate the expected exchange, in the presence of DF at 5 mM Mg^{2+} and 30°. Other laboratories have also reported limited exchange (see introductory statement; Azzam *et al.*, 1972) with similarly isolated ribosomal particles. The factors responsible are under investigation.

Subramanian and Davis (1973) recently reported that when post-runoff exchange of subunits is avoided polysome runoff can be shown to release intact free 70S ribosomes. These have not passed through a pool of free subunits, but they can be expected to equilibrate rapidly with such a pool, at a low concentration (Infante and Baierlein, 1971). The present paper shows that further net dissociation of the ribosomes by IF_3 (either pure or as crude IF), yielding 50S subunits and $30S \cdot IF_3$ complexes, also involves a rapidly reversible equilibrium. This spontaneous reversibility provides further evidence against the suggestion (Kaempfer, 1973) that the free 70S ribosome is a storage form, appearing only under conditions of impaired protein synthesis.

References

Azzam, M. E., Perazzolo, C. A., and Algranati, I. D. (1972), *FEBS (Fed. Eur. Biochem. Soc.) Lett. 21*, 165.

Beller, R. J., and Davis, B. D. (1971), *J. Mol. Biol. 55*, 477.

Davis, B. D. (1971), *Nature (London)*, *231*, 153.

Davis, B. D., and Mingioli, E. S. (1950), *J. Bacteriol. 60*, 17.

Dubnoff, J. S., and Maitra, U. E. (1971), *Methods Enzymol. 20*, 248.

Fiske, C. H., and Subbarow, Y. (1925), *J. Biol. Chem. 66*, 375.

Infante, A. A., and Baierlein, R. (1971), *Proc. Nat. Acad. Sci. U. S. 68*, 1780.

Iwasaki, K., Sabol, S., Wahba, A. J., and Ochoa, S. (1968), *Arch. Biochem. Biophys. 125*, 542.

Kaempfer, R. (1970), *Nature (London) 228*, 534.

Kaempfer, R. (1971), *Proc. Nat. Acad. Sci. U. S. 68*, 2458.

Kaempfer, R. (1973), *J. Mol. Biol. 71*, 583.

Parenti-Rosina, R., Eisenstadt, A., and Eisenstadt, J. M. (1969), *Nature (London) 221*, 363.

Sabol, S., Meier, D., and Ochoa, S. (1973), *Eur. J. Biochem. 33*, 332.

Sabol, S., and Ochoa, S. (1971), *Nature (London), New Biol. 234*, 233.

Sabol, S., Sillero, M. A. G., Iwasaki, K., and Ochoa, S. (1970), *Nature (London) 228*, 1269.

Subramanian, A. R. (1972), *Biochemistry 11*, 2710.

Subramanian, A. R., and Davis, B. D. (1970), *Nature (London) 228*, 1273.

Subramanian, A. R., and Davis, B. D. (1971), *Proc. Nat. Acad. Sci. U. S. 68*, 2453.

Subramanian, A. R., and Davis, B. D. (1973), *J. Mol. Biol. 74*, 45.

Subramanian, A. R., Davis, B. D., and Beller, R. J. (1969), *Cold Spring Harbor Symp. Quant. Biol. 34*, 224.

Subramanian, A. R., Ron, E. Z., and Davis, B. D. (1968), *Proc. Nat. Acad. Sci. U. S. 61*, 761.

Zitomer, R. S., and Flaks, J. G. (1972), *J. Mol. Biol. 71*, 263.

Collapsed Structure Polymers. A Scattergun Approach to Amino Acid Copolymers†

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ABSTRACT: A scheme is proposed for surveying the requirements of producing nonnatural sequence, globular, space-filling polyamino acids based on copolymerization and a double chromatographic procedure. Both experimental and theoretical considerations are used to show that the chromatographic technique can distinguish material having significant tertiary structure from material having only secondary structure. However, in a conformationally heterogeneous mixture the extent of tertiary structure and solvent exclusion is not readily deduced without auxillary measurements. A brief survey of several preparations is reported. A terpolymer containing equimolar amounts of lysine and glutamic acid plus 40 mol % alanine gives good evidence of having significant amounts of collapsed or partially collapsed material at neutral pH. Finally, arguments are made which suggest that the sequence requirements for globular formation deduced from studies on contemporary proteins may be overly strict.

Globular proteins and enzymes in biologically active conformations are unique macromolecular copolymers in that they apparently exist as collapsed, space-filling structures while molecularly dispersed in solution. Studies on wet crystals of globular proteins by X-ray diffraction indicate that they have a well-defined though irregular surface in contact with solvent (Blake, 1972; Drenth *et al.*, 1971; Quiocho and Lipscomb, 1971). The nonpolar amino acid side chains tend to be partially or completely removed from contact with solvent. Internal solvent when present is localized and not like bulk solvent. When molecularly dispersed in solution, hydrodynamic (Tanford, 1961; Yang, 1961) and chromatographic (Ackers, 1970) studies indicate the globular structure is maintained, while many types of studies indicate that some residues are not in contact with solvent (Roberts and Jardetzky, 1970; Tanford, 1962a, 1968; Wetlaufer, 1962). Any agent which destroys the globular integrity of a protein either destroys or greatly reduces the biological activity. As biological activity is sometimes lost with no apparent change in gross molecular conformation, loss of globular integrity is more than a sufficient condition for loss of activity in contemporary proteins and enzymes. By contrast, most synthetic polymers (Flory, 1953) as well as "completely denatured" proteins (Lapanje and Tanford, 1967; Tanford *et al.*, 1966, 1967) exist in solution as polymer random coils, in which most of the polymer domain is occupied by mobile, bulklike solvent and the entire monomeric unit has contact with solvent. The remaining type of macromolecular conformation in solution, an ordered structure such as a helix or a pleated sheet, has solvent in contact with at least part of each monomeric unit. The exclusion of solvent while molecularly dispersed is thus unique to globular proteins. Although there is considerable evidence that the globular or space-filling conformation is an important aspect of the biological, particularly catalytic, activity of proteins, this is not to say that a globular conformation is a static struc-

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