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Binding of Dihydrostreptomycin to *Escherichia coli* Ribosomes: Kinetics of the Reaction

F. N. CHANG AND JOEL G. FLAKS

Department of Biochemistry, University of Pennsylvania Medical School, Philadelphia, Pennsylvania 19104

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Investigations were carried out on the binding of dihydrostreptomycin to purified (and reassociated) 70*S* ribosomes and 30*S* subunits from streptomycin-susceptible strains, and the results were compared with those of similar studies with native (run-off) 70*S* ribosomes. At 0 C, only a small fraction of purified 70*S* ribosomes and 30*S* subunits bound 1 molecule of the antibiotic tightly, and at a rate comparable to the binding occurring with native 70*S* ribosomes. At temperatures of 10 C and above, there was a temperature-dependent increase in the extent of antibiotic binding to purified 70*S* and 30*S* particles up to a maximum of 1 molecule/ribosomal particle, but the kinetics of binding was slow in comparison to that taking place at 0 C. These and other results suggest that a major fraction of 30*S* subunits and purified (or reassociated) 70*S* ribosomes are inactive in binding the antibiotic. This has been localized to an instability of the free 30*S* subunit, which in solution at 0 C has a half-life of 5 hr or less. Inactive 30*S* or 70*S* particles could be thermally activated, with the latter being identical in their streptomycin-binding properties to native 70*S* ribosomes. The activation kinetics were slow in comparison to the binding kinetics for the antibiotic and were indicative of a conformational change in ribosomal structure. There thus appears to be a reversible transition between active and inactive forms of the ribosomal particles for streptomycin binding, but additional binding sites for the antibiotic are not created by the transitions. The active form of the 30*S* subunit can be stabilized in the presence of polyuridylic acid, but much more effectively by association with the 50*S* subunit to form a 70*S* ribosome. The kinetics of dihydrostreptomycin binding were studied in both directions of the reaction, and the reaction in the direction of binding was found to be several orders of magnitude faster than that of the reverse, or debinding, direction. The kinetics of the exchange of bound dihydrostreptomycin with the free antibiotic were also determined and shown to have rate constants that are very similar to those of the debinding reaction, which is the rate-limiting step. It appears likely that the exchange reaction is proceeding via the same reaction pathway. The temperature dependence of the kinetics of dissociation of the bound complex was much greater than that in the direction of binding and accounted for most of the temperature dependence of the binding equilibrium. From the determined thermodynamic and activation parameters, it appears likely that binding of the antibiotic induces a conformational change in ribosomal structure to one that is less ordered than the native particle. Heterogeneity has been found in the kinetics of binding and of exchange, with a fraction of the 70*S* population showing slower kinetics for both directions of the reaction.

In the preceding paper (2), the general characteristics and affinity of the ribosomal binding of dihydrostreptomycin (DSM) were reported. These affinity measurements were made on a variety of ribosomal preparations, but most of the evidence described was obtained with native (run-off) 70*S* ribosomes from SM^S (phenotypically susceptible to streptomycin) strains, because experience had demonstrated it to be the least complicated system. The present report deals with the more complicated binding situation that obtains with purified 70*S* ribosomes and with 30*S* subunits. Vogel et al. (16) reported that the isolated 30*S* subunit is unstable in solution with regard to its ability to bind DSM, and that there are conditions which will stabilize the active conformation of the subunit. We have confirmed these observations and have also found that inac-

tive 30S subunits and purified 70S ribosomes can be thermally activated in a manner suggestive of a conformational change in structure.

In addition, the kinetics of the binding of DSM and of the dissociation of the DSM-ribosome complex were studied, as well as the kinetics of the exchange of bound DSM. A more complete thermodynamic picture of the binding has thus been obtained that suggests a conformational change in ribosome structure resulting from the binding of the streptomycin (SM) antibiotics.

MATERIALS AND METHODS

All of the ribosomal preparations were obtained from two SM^S K-12 strains of *E. coli*, Q13 and JC-355, both of which show identical DSM-binding properties. The materials and all preparative and assay procedures were identical to those described in the preceding paper (2).

RESULTS

General characteristics of ^{3}H-DSM binding to purified 70S ribosomes and 30S subunits. In contrast to the results obtained with native (run-off) 70S ribosomes from SM^S strains where the binding of exactly 1 molecule of ^{3}H-DSM per ribosome was essentially temperature-independent and very rapid (2), similar studies with purified 70S ribosomes and 30S subunits exhibit notable differences in regard to rate and temperature dependence. The binding kinetics at several different temperatures for purified 70S ribosomes are shown in Fig. 1 and for 30S subunits in Fig. 2. The 70S preparation was obtained via the ammonium sulfate precipitation procedure of Kurland (8), but identical results were obtained with ribosomes carried through any high-salt preparative procedure. Qualitatively, the results in both Fig. 1 and 2 are similar, but there are marked quantitative differences. In general, the binding to 30S subunits was much more temperature-dependent and slower than the corresponding binding to purified 70S ribosomes, and the ultimate extent of binding with prolonged incubation (i.e., the number of particles which bind 1 molecule of DSM) was always lower with the 30S subunit. After incubation for 60 min at 37 C, a plateau value of 0.85 molecule of ^{3}H-DSM bound/ribosome was found with purified 70S particles, and there was no further increase in binding with extended incubation periods. After corrections are applied as indicated in the preceding paper (2), this corresponds to the binding of exactly 1 molecule/ribosome for those capable of binding (~95% of the total ribosomes).

When polyuridylic acid (poly U) was present at 37 C, the kinetics of binding were faster and the maximal binding was obtained after 10 min

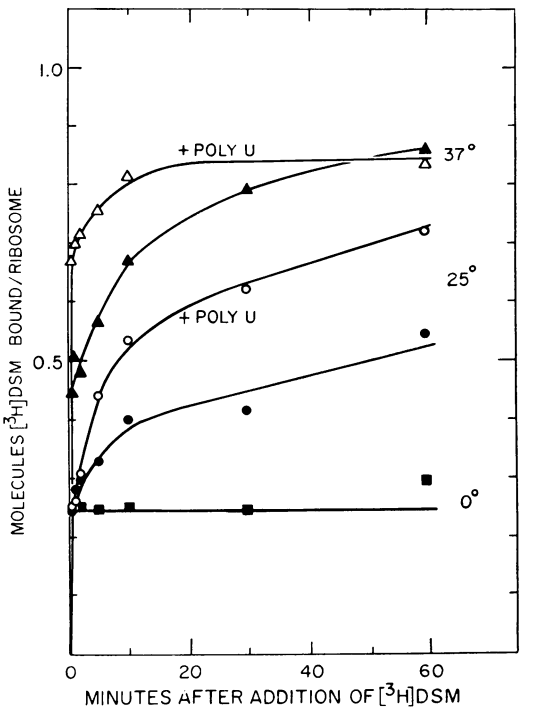

FIG. 1. *Kinetics of the binding of ^{3}H-dihydrostreptomycin (DSM) to purified 70S ribosomes and the effect of poly U. The incubations were carried out in a total volume of 0.2 ml containing: standard assay buffer; purified 70S ribosomes (strain JC-355), 3.6 × 10^{-7} M (3.0 A_{260} units); and ^{3}H-DSM, 3.4 × 10^{-6} M (1.88 × 10^5 counts/min). The concentration of poly U, where added, was 2.5 μg per 0.2-ml reaction mixture. The reaction mixtures without poly U were incubated at 0 C (■), 25 C (●), and 37 C (▲); those containing poly U were incubated at 25 C (○) and 37 C (△) for the times indicated, diluted to 2.5 ml with cold standard assay buffer, and immediately carried through the filtration assay. A blank was determined by adding the same amount of ^{3}H-DSM to the assay buffer without ribosomes and filtered as above. This blank was less than 40 counts/min. The binding of 1 molecule of ^{3}H-DSM per ribosome = 15,600 counts/min.*

of incubation, again with no further increase with extended incubation. Similar stimulatory effects of poly U could be seen at 25 C; however, at temperatures of 10 C or less, no significant stimulation of binding was found. The stimulatory effect of poly U was variable with different ribosome and subunit preparations, and was always greater with 70S ribosomes than with 30S subunits. Among the homopolynucleotides, the largest stimulatory effect has been found with poly U, in confirmation of previous reports (7). In our studies, however, the magnitude of the

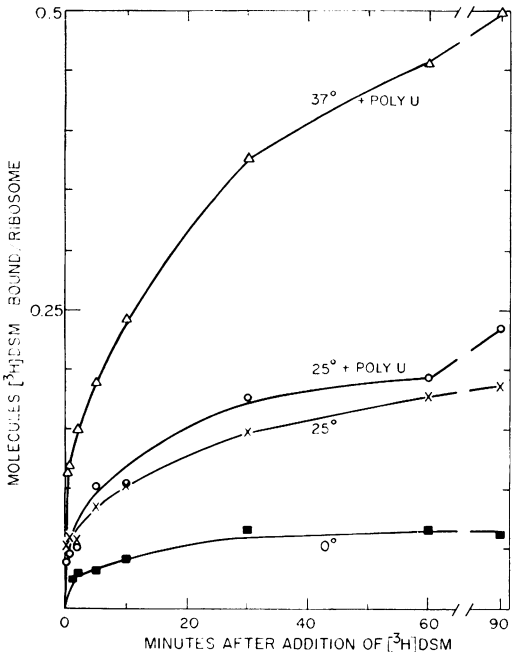

FIG. 2. *Kinetics of the binding of ^{3}H-dihydrostreptomycin (DSM) to purified 30S ribosomal subunits. The reaction mixtures were the same as those described in Fig. 1 except that the concentration of 30S subunits (strain JC-355) was 2.3 × 10^{-7} M (0.64 A_{260} units) and that of ^{3}H-DSM was 3.4 × 10^{-6} M (1.88 × 10^5 counts/min). The reaction mixtures were incubated for the times indicated at 0 (■) and 25 C (×) in the absence of poly U and at 25 (○) and 37 C (△) in the presence of 2.5 μg of poly U per 0.2-ml reaction mixture. One molecule of ^{3}H-DSM bound/30S subunit = 10,000 counts/min.*

stimulatory effect was always much smaller than that previously reported (7).

The concentration dependence on ^{3}H-DSM for its binding to purified 70S ribosomes at 37 C is shown in Fig. 3. These results are qualitatively similar to an identical experiment carried out with 30S subunits (see Fig. 7 of the preceding paper [2]). Throughout most of the range of ^{3}H-DSM concentrations up to 10^{-5} M, the extent of binding in the presence of poly U was greater than in its absence, but in no instance was it greater than 1 molecule of DSM bound/ribosome. Although not shown, with more extended times of incubation in the absence of poly U, a family of curves was generated which progressively approached that of the incubation in the presence of poly U. Taken together with the results of Fig. 1 and 2, the evidence suggests that the poly U stimulation is a kinetic effect; poly U most certainly does not create an additional binding site on the 30S or 70S particle. It can also be noted in Fig. 3 that the apparent binding affinity was approximately the same in the presence or absence of poly U, with a K_{diss} in the vicinity of 10^{-7} M.

At 0 C, a fraction of the purified 70S ribosomes and 30S subunits bound ^{3}H-DSM as rapidly as native 70S particles (Fig. 1 and 2). This fraction varied in amount in different ribosome preparations, ranging from 15 to 40% of the total in purified 70S preparations and from 3 to 8% in 30S subunit preparations. The lower limit in the latter case is at the experimental error of the binding assay and may be indicative of zero activity in some 30S preparations.

The above results confirm and extend the studies reported by Vogel et al. (16), whose model we favor: the active conformation of the isolated 30S subunit appears to be unstable in solution, with one manifestation of this being a loss in the ability to bind DSM. Any transitory treatment of native 70S ribosomes which will free the 30S subunit, as in any of the high-salt preparative procedures, will result in a fraction of the 70S particles being inactivated for the binding of DSM. Both inactive 30S subunits and 70S ribosomes can be thermally activated in a reaction which is slow kinetically in comparison to the kinetics of DSM binding. It was further suggested that thermally activated 30S subunits can be stabilized in the presence of poly U or, as shown here, more effectively by association with the 50S subunit.

This interpretation of the poly U effect is

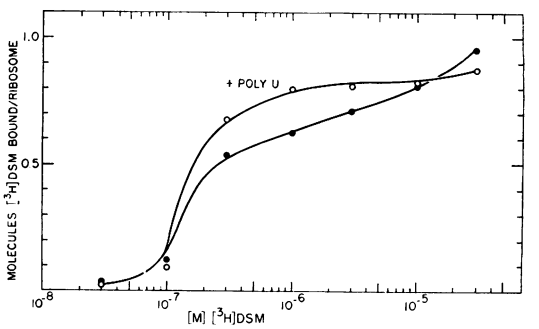

FIG. 3. *Concentration dependence of ^{3}H-DSM for binding to purified 70S ribosomes. The reaction mixtures were the same as those described in Fig. 1 except that the concentration of purified 70S ribosomes (strain JC-355) was fixed at 2.5 × 10^{-7} M (2.1 A_{260} units) and the concentrations of ^{3}H-DSM varied from 1.5 × 10^{-8} to 3 × 10^{-5} M. The concentration of poly U, where added, was 2.5 μg per 0.2 ml of reaction mixture. The reaction mixtures were incubated at 37 C in the absence (●) or in the presence of poly U (○) for 30 min, and were assayed by the filtration procedure as indicated in Fig. 1. One molecule of ^{3}H-DSM bound per ribosome = 10,850 counts/min.*

markedly different from that proposed by Kaji and Tanaka (7; H. Kaji and Y. Tanaka, Fed. Proc. **28**:865, 1969), who discovered the effect. These investigators argued for the creation of an additional binding site upon the interaction of poly U and the 30*S* subunit, but no evidence has been found for this. After prolonged incubation at higher temperatures, the extent of binding is the same in the presence or absence of poly U and approaches, but does not exceed, 1 molecule of DSM/70*S* ribosome or 30*S* subunit. Also, at temperatures below 10 C, where the association of poly U and the subunit still occurs, there is no stimulatory effect on DSM binding. Finally, although the poly U effect can be demonstrated with purified 70*S* ribosomes, it is completely absent with native 70*S* particles, and these two types of 70*S* particles are interconvertible in their DSM-binding properties without the intervention of poly U.

Reversible transition between ribosomes active and inactive in binding ^{3}H-DSM. Further evidence in support of the proposed interpretation is the demonstration of the reversible transition of particles active and inactive in binding DSM and the separation of the transition phenomena from the binding reaction itself. The latter was accomplished by testing for the binding of ^{3}H-DSM at 0 C, where active particles will bind the antibiotic rapidly but where the thermal activation is suppressed. The evidence is summarized in Tables 1 and 2, in which all of the binding assays were carried out at 0 C.

In experiment 1 of Table 1, the dissociation of native 70*S* ribosomes to subunits by dialysis against 0.1 mM Mg^{2+}, followed by their reassociation to 70*S* particles in 10 mM Mg^{2+}, resulted in an 84% loss in the ability of the reassociated particle to bind DSM when compared with the native 70*S* ribosome from which it was derived. The residual binding ability is now almost identical to that of the purified 70*S* ribosome (experiment 2), and in addition, although not shown here, the kinetics of binding with these reassociated 70*S* ribosomes and its stimulation by poly U was also identical to that obtained with purified 70*S* ribosomes (Fig. 1). The ability to bind DSM with the same kinetics and to the same extent as a native 70*S* ribosome could be almost quantitatively restored to both reassociated or purified 70*S* ribosomes by heating either preparation at 37 C for 30 min (experiments 1 and 2). Optimal activation could also be obtained with shorter incubation times at higher temperatures (10 min at 45 C or 5 min at 50 C).

The results presented in experiment 3 of Table 1 demonstrate that it is the 30*S* subunit which is thermally activated; no activation was found with the 50*S* subunit. There are two other points to be noted here. Firstly, the incubation conditions for 30*S* subunit activation (37 C for 60 min) are optimal values, and thus the 30*S* subunit requires a longer incubation than that required

TABLE 1. *Effect of dissociation, reassociation, and preheating on binding of ^{3}H-dihydrostreptomycin (DSM) to ribosomes or subunits*[a]

Expt	Ribosome source (and pretreatment)	Molecules of ^{3}H-DSM bound/ribosome or subunit
1	Native 70*S*	0.85
	Native 70*S* (dialyzed vs. 0.1 mM Mg^{2+} then reassociated in 10 mM Mg^{2+}	0.14
	Reassociated 70*S*[b] (preheated at 37 C for 30 min)	0.78
2	Native 70*S*	0.83
	Purified 70*S*	0.16
	Purified 70*S* (preheated at 37 C for 30 min)	0.80
3	30*S*	0.04
	50*S*	0.0
	30*S* + 50*S*	0.06
	30*S* (preheated at 37 C for 60 min)	0.38
	30*S* (preheated at 37 C for 60 min) + 50*S*	0.52
	50*S* (preheated at 37 C for 60 min)	0.01
	50*S* (preheated at 37 C for 60 min) + 30*S*	0.06
	30*S* + 50*S* (both preheated separately at 37 C for 60 min, then mixed)	0.56

[a] All ribosome and subunit preparations were from strain Q13. The preheating step, where indicated, was carried out in standard assay buffer and the samples were chilled to 0 C immediately thereafter. All incubations for the assay of ^{3}H-DSM binding were carried out at 0 C for 10 min. The concentrations of reactants for experiments 1 and 2 were identical to those of Fig. 1. In experiment 3, the assay for ^{3}H-DSM binding was carried out in a total volume of 0.11 ml, containing: standard assay buffer, 4.3×10^{-6} M 30*S* or 50*S* subunits (0.65 A_{260} units for 30*S* or 1.3 A_{260} units for 50*S*), and 6.3×10^{-6} M ^{3}H-DSM (1.36×10^5 counts/min). The binding of 1 molecule of ^{3}H-DSM per ribosome = 15,600 counts/min for experiments 1 and 2, and 10,200 counts/min for experiment 3.

[b] Native 70*S* dialyzed against 0.1 mM Mg^{2+} and then reassociated in 10 mM Mg^{2+}.

TABLE 2. *Stability of the activated 30S subunit for ^{3}H-dihydrostreptomycin (DSM) binding*[a]

System	Molecules of ^{3}H-DSM bound/ribosome or subunit			
	(a) 0 C for 60 min	(b) 37 C for 60 min	(c) (b) + 0 C for 24 hr	(d) (c) + 37 C for 60 min
30S	0.06	0.41	0.12	0.29
50S	0.0	0.01	0.0	0.01
30S + 50S	0.12	0.76	0.19	0.51
Purified 70S	0.16	0.84	0.83	0.83

[a] The ribosome and subunit preparations were from strain Q13, and the conditions of pretreatment are indicated under a through d. The system 30S + 50S represents subunits taken through the pretreatment separately and then mixed just prior to the assay for ^{3}H-DSM binding. ^{3}H-DSM binding was assayed at 0 C as indicated in experiment 3 of Table 1.

for the optimal activation of the reassociated or purified 70S ribosome (experiments 1 and 2). Secondly, the extent of activation of the 30S subunit is only about 70% of that obtained on reactivating the 70S particle. These two observations are believed to be manifestations of the instability of the activated 30S subunit when the particle is free in solution, and direct evidence bearing on this is presented in Table 2. After optimal thermal activation, as indicated in column b, if activated 30S subunits were allowed to stand at 0 C for 24 hr (column c), then approximately 70 to 75% of the activated DSM-binding ability was lost. A part, but not all, of this lost activity could be restored by a second cycle of thermal activation (column d). Preliminary studies have indicated that the half-life of the active 30S subunit at 0 C is 5 hr or less. In contrast to the above, if the 30S subunit was already associated with the 50S subunit (purified 70S) prior to thermal activation, there was no loss in DSM-binding activity on standing at 0 C. To a lesser extent, the same stabilizing effect could be obtained with poly U.

Taken together with the previous results of Table 1 and the kinetic studies of Fig. 1 and 2, a consistent picture of a reversible transition is apparent. No evidence has yet been found with either the 30S or 70S particles for changes in sedimentation as a result of the transition phenomenon. The possibility that dimerization of the particles is responsible for the phenomenon has been ruled out, and the evidence and arguments for this are cited in the Discussion section of the preceding paper (2).

Influence of Mg^{2+} on the stability of the 70S-^{3}H-DSM complex. Studies were carried out on the stability of the 70S-^{3}H-DSM complex to changes in the Mg^{2+} environment. Mg^{2+} at a concentration of 10 mM was shown in the preceding paper (2) to be an absolute requirement for the binding of DSM to either 30S or 70S particles; however, it was not known from these studies whether there was a Mg^{2+} requirement for maintenance of the bound complex. Interest in this question stems from the observation originally made by Herzog (5), and extended by Luzzatto et al. (10), that the bulk of the ribosomes extracted from bacteria killed by the antibiotic are 70S particles, formed apparently at the expense of the pool of free subunits and polysomes. These particles have been referred to as "stuck 70S" particles. The results of an experiment directed at this question are presented in Fig. 4. The 70S-^{3}H-DSM complex was generated via a

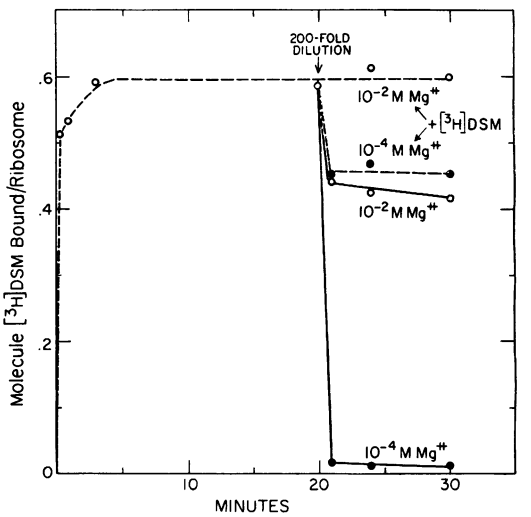

FIG. 4. *Stability of the 70S-^{3}H-dihydrostreptomycin (DSM) complex. The incubations were carried out in a total volume of 0.25 ml containing: standard assay buffer; native 70S ribosomes (strain JC-355), 1.2×10^{-6} M (12.5 A_{260} units), and ^{3}H-DSM, 7.8×10^{-6} M (5.25×10^5 counts/min). Samples (10 μliters) were withdrawn after incubation at 25 C for 0.5, 1, 3, 20, 24, and 30 min, diluted to 0.5 ml with cold assay buffer, and filtered (○, dashed line). At 20 min, 40-μliter samples were withdrawn from the original incubation mixture and diluted to 8 ml with standard assay buffer containing either 10 mM Mg^{2+} (○, solid line) or 0.1 mM Mg^{2+} (●, solid line). A 40-μliter sample from the original incubation mixture was also diluted 200-fold into the standard assay buffer containing 0.1 mM Mg^{2+} but with the final concentration of ^{3}H-DSM fixed at 9×10^{-8} M (●, dashed line). Incubations were continued at 25 C, and 2-ml samples were withdrawn and carried through the filtration assay. One molecule of ^{3}H-DSM bound per ribosome = 3,000 counts/min.*

20-min incubation, after which a series of 200-fold dilutions were made from the incubation mixture. One of these dilutions was made into the standard assay buffer containing 10 mM Mg^{2+}, and a second dilution was made into the same buffer modified to contain 0.1 mM Mg^{2+}. The remaining pair of dilutions were made into the same solutions indicated above, but in addition they were supplemented with ^{3}H-DSM such that the final concentration of the antibiotic (0.9×10^{-7} M, including the contribution from the bound complex) was very slightly below the K_{diss} of the bound complex (0.94×10^{-7} M).

When the dilution was made into 10 mM Mg^{2+}, there was a loss of approximately one-third of the bound complex, whereas on dilution into 0.1 mM Mg^{2+} all of the bound ^{3}H-DSM was rapidly lost. The loss of one-third of the bound complex by dilution into 10 mM Mg^{2+} was close to the expectation resulting from the shift in the equilibrium by dilution, and the conclusion from this part of the experiment is that Mg^{2+} is required for the stability of the 70*S*-DSM complex.

However, if the dilution was made into the same pair of buffers supplemented with a ^{3}H-DSM concentration near the K_{diss} value, there was the unexpected finding that in 0.1 mM Mg^{2+} about 75% of the bound complex did not immediately dissociate. Although the amount lost satisfies the equilibrium requirements, the presence of the remaining bound fraction implies that 10 mM Mg^{2+} is no longer necessary to maintain the bound form and that it can exist in 0.1 mM Mg^{2+}. The bound form in this latter instance cannot be a 30*S*-^{3}H-DSM complex, because K_{diss} for the 30*S*-^{3}H-DSM complex is 10-fold higher than that for the 70*S* complex, and accordingly the residual amount bound should have been reduced to below 10% of the original level after the dilution. Results similar to those shown in Fig. 4 could be obtained with lower concentrations of ^{3}H-DSM, but the concentration could not be taken very far below the K_{diss} value of the 70*S*-^{3}H-DSM complex. There is a rather strong implication that the equilibrium for DSM binding to the 70*S* ribosome is more important for the stability of the complex than the involvement of Mg^{2+}, and this is somewhat difficult to reconcile with the known requirement of 10 mM Mg^{2+} for the forward (binding) step of the equilibrium. The fraction which is stable in 0.1 mM Mg^{2+} has suggested itself as analogous to, or perhaps identical with, the putative "stuck 70*S*" particle, but thus far all attempts to demonstrate such a particle by zone sedimentation have been unsuccessful. Herzog (5) was also unable to demonstrate the formation of "stuck 70*S*" particles in vitro, and the only means of obtaining them is by extraction from SM-killed bacterial cells.

Kinetics of the formation and dissociation of the 70S-^{3}H-DSM complex and the exchange of bound ^{3}H-DSM. A preliminary study of the kinetics of the forward (binding) and reverse (debinding) reactions comprising the equilibrium for the formation of the 70*S*-DSM complex (equation 1),

$$70S + \text{DSM} \underset{k_{-1}}{\overset{k_1}{\rightleftharpoons}} 70S\text{-DSM} \qquad (1)$$

revealed that the debinding reaction was slower by several orders of magnitude, and it could be quantitated far more precisely than the forward binding reaction. In these studies, the formation of the 70*S*-^{3}H-DSM complex was allowed to proceed until an equilibrium was established, and after an extensive dilution, the rate of disappearance of the bound complex was determined. The results of such studies over the temperature range from 3 to 25 C are shown in Fig. 5. As expected from equation 1, the debinding reaction displayed first-order kinetics with an increase in rate as the temperature was increased. Surprisingly, however, the rate plots were biphasic throughout the temperature range studied.

The exchange of ribosome-bound ^{3}H-DSM with free DSM was first reported by Kaji and Tanaka (7), and we have shown in the preceding paper (2) that the specificity of this reaction is in complete accord with the biological activity of the SM antibiotics. Kinetic studies of the exchange reaction can be approached by the same procedure as that described above for determining the rate of the debinding reaction by replacing the dilution step with a simple addition of a known amount of unlabeled DSM. The results of such studies, at 3 and 25 C, are presented in Fig. 6. Theoretical exchange represents the limit of radioactivity expected in the complex and is calculated from the known dilution in specific activity of the ^{3}H-DSM. The exchange reaction is temperature-dependent, and the experimental values approach the theoretical exchange after 6 min at 25 C and 100 min at 3 C. As shown in Fig. 7, the exchange reaction also obeyed first-order kinetics, again with a biphasic response indicative of heterogeneity.

Rate constants have been calculated for both the fast- and slow-reacting species from the first-order plots of Fig. 5 and 7. These values are tabulated in Table 3 in the two columns under k_{-1}. In addition, $t_{1/2}$ values for these reactions were calculated and are listed in Table 3. It has been the general finding with these and other data that the rates of the debinding and exchange reactions are very similar, although not identical. Values of the rate constants for the exchange reaction

are usually between 13 and 20% lower than those for the debinding reaction throughout the temperature range studied. The reason for this difference, although it is small, is not readily apparent, but there are greater experimental uncertainties in these measurements. The similarity between the rate constants for the debinding and exchange reactions and the fact that the debinding reaction is the rate-limiting one in establishing the equilibrium strongly suggest that the exchange reaction is proceeding via the same reaction pathway that is involved in establishing the equilibrium. However, such rate measurements by themselves are not unequivocal proof of this suggestion.

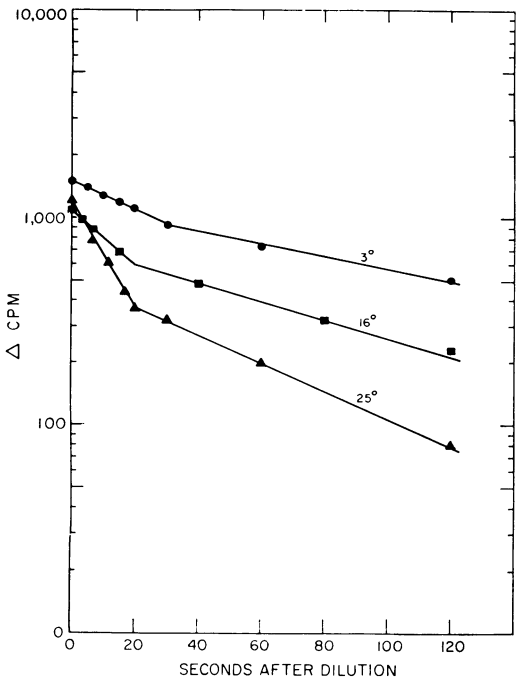

FIG. 5. *Kinetics of the dissociation of the 70S-^{3}H-dihydrostreptomycin (DSM) complex. The initial incubation was carried out in a total volume of 0.6 ml containing: standard assay buffer; native 70S ribosomes (strain JC-355), 1.9 × 10^{-6} M (48 A_{260} units); and ^{3}H-DSM, 6.8 × 10^{-6} M (1.13 × 10^6 counts/min). After incubation for 15 min at 25 C to allow formation of the 70S-^{3}H-DSM complex, 0.15-ml samples were withdrawn and diluted with 30 ml of standard assay buffer (200-fold dilution) at the respective temperature. The incubations were continued at 3 (●), 16 (■), and 25 C (▲). Samples of 3 ml were removed at various times and filtered. Owing to the limiting capacity of the nitrocellulose filter to adsorb ribosomes (up to 4 A_{260} units), zero time points were obtained by diluting 50-μliter samples of the original incubation mixture with 1 ml of standard buffer (20-fold dilution) so that the concentration of free ^{3}H-DSM was still far above the K_{diss} of the 70S-^{3}H-DSM complex (which is 0.94 × 10^{-7} M at 25 C). These values at 3 and 25 C were 3,720 and 3,340 counts/min, respectively. A new equilibrium was reached after a 200-fold dilution at all three temperatures. The time required to reach this equilibrium varied with temperature, 20 min at 3 C and less than 5 min at 25 C. The equilibrium values were 2,221 and 2,122 counts/min at 3 and 25 C, respectively. The plotted values at the different times (Δ counts/min) after dilution represents the difference between the observed counts/min and the counts/min at equilibrium [(counts/min)$_t$ − (counts/min)$_{eq}$].*

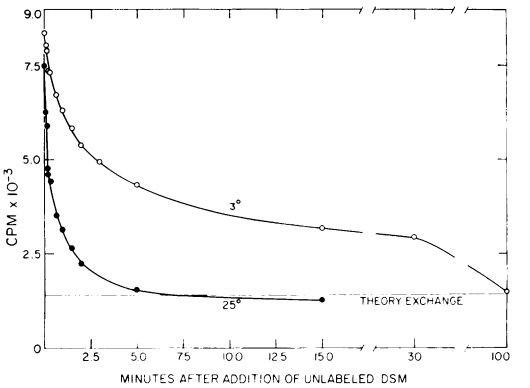

FIG. 6. *Kinetics of the exchange of bound ^{3}H-dihydrosteptomycin (DSM). The initial incubations were carried out in a total volume of 3.0 ml containing: standard assay buffer; native 70S ribosomes (strain JC-355), 3.2 × 10^{-7} M (40 A_{260} units); and ^{3}H-DSM, 2.2 × 10^{-6} M (1.88 × 10^6 counts/min). After incubation at 3 or 25 C for 15 min, a series of 0.2-ml samples were quickly diluted with 2 ml of standard assay buffer containing 2 nmoles of unlabeled DSM (5.6-fold dilution with respect to ^{3}H-DSM), and the incubations were continued at the respective temperatures. At the times indicated in the figure, the entire contents of the 2-ml samples were filtered. The zero time samples were diluted with 2.0 ml of standard assay buffer containing no unlabeled DSM. The incubation temperature was 3 (○) or 25 C (●). The binding of 1 molecule of ^{3}H-DSM per ribosome = 13,000 counts/min. The theoretical exchange equilibrium is calculated from the 5.6-fold dilution in ^{3}H-DSM specific activity and the zero time binding of ^{3}H-DSM.*

From the known values of K_{diss} for the binding equilibrium (2) and the values for k_{-1} from Table 3, it is possible to calculate values for the second-order rate constant, k_1, for the forward reaction. This follows from the relationship: $K_{diss} = k_{-1}/k_1$. Calculated values for k_1 are listed in Table 3. It is extremely difficult to determine k_1 directly with the available methodology owing to the rapidity of the binding reaction, but several attempts at this have given values which are in reasonable accord with the calculated values in Table 3. For the fast-reacting species, the directly determined k_1 in units of

M^{-1} sec^{-1}, is 2.5 × 10^5 at 3 C and 3.5 × 10^5 at 25 C. These values are 36 and 38% lower, respectively, than the corresponding calculated values in Table 3. This provides some direct assurance of the validity of the calculated values of k_1 in Table 3, but the latter are believed to be more accurate and have been used for the calculation of the activation parameters of Table 5.

The evidence for heterogeneity presented in Fig. 5 and 7 has also been found in a direct study

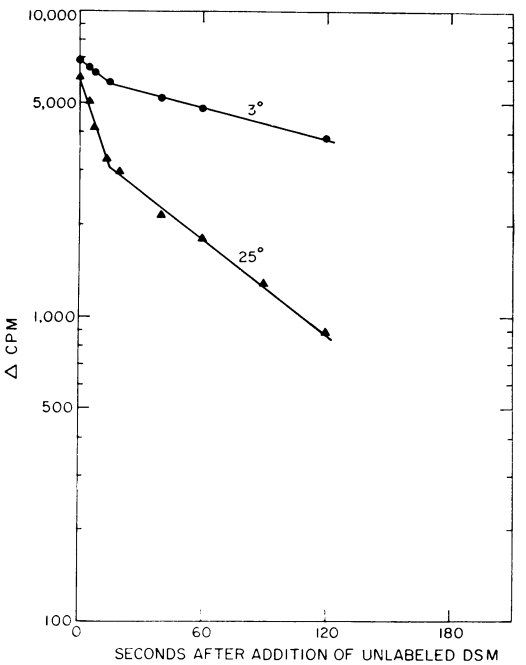

FIG. 7. *Evaluation of rate constants for the exchange of bound ^{3}H-dihydrostreptomycin (DSM). First-order plot of the data from Fig. 6. The Δ counts/min was obtained by subtracting the counts/min of the theory exchange from the observed value of counts/min and plotted against time as in Fig. 5.*

of the forward reaction. Thus, there is direct evidence for this in both directions of the equilibrium. An estimate of the fraction of the ribosomal population comprising the fast- and slow-reacting species can be obtained by extrapolation of the rate plots for the slow-reacting species in Fig. 5 and 7 to the ordinate value. The calculated distribution for both species is given in Table 4 for the temperature range studied. For the fast species, the percentage ranged from 15 to 25% of the total at 3 C and increased to 45 to 58% of the total at 25 C. Thus, the distribution of both species in the ribosomal population is temperature-dependert. The heterogeneity demonstrated here is not a function of the purity of the ribosome, in so far as this has been examined to date. Although the data from Fig. 5 and 7 were obtained with native 70*S* ribosomes, a similar heterogeneity was found with purified 70*S* ribosomes (high salt) that were thermally activated (90% active) prior to the kinetic study. Also, in the large number of studies on the equilibrium of the binding (2), no evidence for such heterogeneity has been found in the Scatchard plots, and from the magnitude of the heterogeneity in the kinetic studies this surely should have been seen. The explanation for this seeming disparity resides in the finding of heterogeneity for the kinetic response in both directions of the equilibrium, with the affinity of all molecules of DSM bound being the same. There is now direct and good evidence for heterogeneity in the 30*S* subunit from studies of both its protein composition and function (9, 15).

Temperature dependence of the reaction kinetics. Arrhenius plots of the logarithm of the rate constants, k_{-1} and k_1, against the reciprocal of the absolute temperature are shown in Fig. 8. The values for the rate constants are those for the fast-reacting species, with k_{-1} directly determined from the debinding reaction kinetics and k_1 calculated as indicated in Table 3. Over the temperature range studied, from 3 to 25 C, there was

TABLE 3. *Summary of the rate constants*

Species	Temp (C)	k_{-1} (sec^{-1})		$t_{1/2}$ (sec)[a]		k_1 (M^{-1} sec^{-1})[b]	
		Dilution	Exchange	Dilution	Exchange	Dilution	Exchange
Fast	3	0.0158	0.0126	44	55	3.9 × 10^5	3.1 × 10^5
	16	0.0315	—	22	—	4.8 × 10^5	—
	25	0.053	0.046	13	15	5.6 × 10^5	4.9 × 10^5
Slow	3	0.0063	0.0041	110	170	0.154 × 10^5	0.10 × 10^5
	16	0.0103	—	67	—	0.157 × 10^5	—
	25	0.0154	0.0126	45	55	0.164 × 10^5	0.134 × 10^5

[a] The value of $t_{1/2}$ is $0.693/k_{-1}$.

[b] The value of k_1 is equal to k_{-1}/K_{diss}, with the values of K_{diss} taken from the preceding paper (2).

TABLE 4. *Estimation of the extent of 70S heterogeneity with regard to ^{3}H-dihydrostreptomycin binding as a function of temperature*[a]

Temp (C)	Percent fast species		Percent slow species	
	From dilution	From exchange	From dilution	From exchange
3	25	15	75	85
16	37	—	63	—
25	58	45	42	55

[a] The percentages are calculated from the data of Fig. 5 and 7 by extrapolation to zero time and estimation of the fraction of each species from the total.

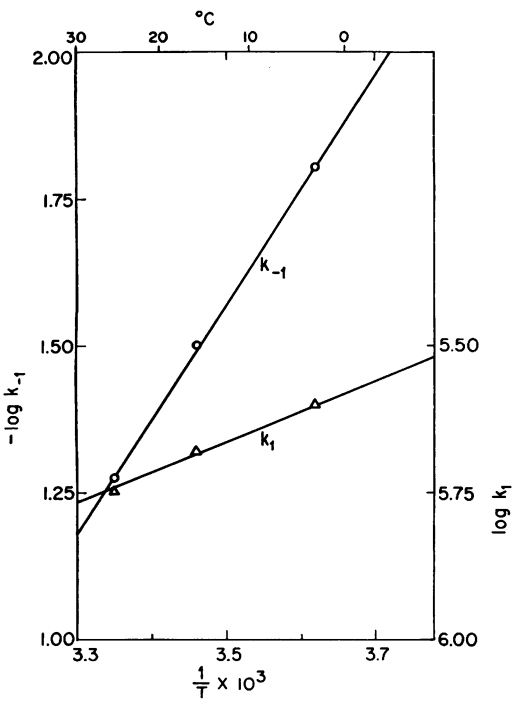

FIG. 8. *Arrhenius plot of the relationship of the rate constants, k_1 and k_{-1}, with temperature. The rate constants for the fast species obtained from Table 3 were used for the plot. The Arrhenius activation energy, E, is obtained from $-(slope) \times 2.303R$.*

linear dependence on temperature for the rate constants for both the forward and reverse reactions, which was also found for K_{diss} in the equilibrium studies (2). The slope for the reverse reaction is 3.3-fold greater than that for the forward reaction, indicative of the greater temperature dependence for activation of debinding. Activation parameters for both the forward and reverse reactions are given in Table 5, along with the corresponding thermodynamic parameters ($\Delta G°$, $\Delta H°$, and $\Delta S°$) for the equilibrium as determined in the preceding paper (2). The values for the rate constants for these calculations are for the fast-reacting species and are the ones used for the Arrhenius plots in Fig. 8. All four activation parameters are of the same sign for both reaction directions. The larger temperature dependence for the reverse reaction is reflected in the fourfold greater enthalpy of activation ($\Delta H^‡$). In regard to the entropy of activation ($\Delta S^‡$), the value of -25.6 entropy units/mole for the forward reaction appears to be within the range found for many bimolecular reactions (6), but that of -37.0 entropy units/mole for the debinding activation appears to be larger than that for a simple first-order dissociation reaction. With the reaction in the reverse direction, going from the bound form to the activated complex, there appears to be an appreciable ordering of the structure involved, as indicated from $\Delta S^‡_{-1}$ and $\Delta S°$.

TABLE 5. *Summary of the thermodynamic and activation parameters*[a]

Parameter	Binding reaction	Debinding reaction	Equilibrium[b]
E (kcal/mole)...	2.7	8.9	
$\Delta G^‡$ (kcal/mole).....	9.7	19.3	$\Delta G° = -9.6$
$\Delta H^‡$ (kcal/mole).....	2.1	8.3	$\Delta H° = -6.2$
$\Delta S^‡$ (entropy units/mole)...	−25.6	−37.0	$\Delta S° = 11.4$
$T\Delta S^‡$ (kcal/mole).....	−7.6	−11.0	$T\Delta S° = 3.4$

[a] The values for E were obtained as indicated in Fig. 8. The values for the other activation parameters were calculated from the following equations (3):

$$\Delta H^‡ = E - RT = E - 0.6$$
$$\Delta G^‡ = -RT \ln kh/k_bT$$
$$\Delta S^‡ = (\Delta H^‡ - \Delta G^‡)/T$$

where k is rate constant for the fast species (Table 3), h is Planck's constant (6.62×10^{-27} erg-second), k_b is the Boltzmann constant (1.37×10^{-16} erg degree^{-1}), R is the gas constant (1.987 cal degree^{-1} mole^{-1}), and T is the absolute temperature (298 K).

[b] The values for the equilibrium are taken from the preceding paper (2).

DISCUSSION

We have dealt here exclusively with the tight binding of 1 molecule of the antibiotic to 30S and

50S particles from SM^S strains, and several insights into the nature of the binding are suggested from the thermodynamic and kinetic data. Clearly, the spontaneity of the overall reaction in the direction of the binding of DSM is due to the fact that the reverse (debinding) reaction is thermodynamically less favorable than the forward (binding) reaction. Consistent with this, the activated complex, $[70S\text{-}DSM]^{\ddagger}$, would appear to bear a closer structural resemblance to the reactant 70S ribosome than it does to the product 70S-DSM complex. We infer this from the low activation enthalpy for binding ($\Delta H^{\ddagger}_{1}$) in contrast to that for debinding ($\Delta H^{\ddagger}_{-1}$) and from the entropic changes. As mentioned previously, the activation entropy for binding ($\Delta S^{\ddagger}_{-1}$) is not unique in sign or magnitude from that observed for many bimolecular reactions (6). However, the activation entropy for debinding ($\Delta S^{\ddagger}_{-1}$) is considerably larger than the expectation for a simple first-order dissociation of the type studied here. It would appear that there is some reordering of the ribosome structure involved in the debinding, and most likely this is to a tighter configuration than that which exists in the 70S-DSM complex. This view suggests that the tight binding of 1 molecule of the antibiotic results in a conformational change, perhaps a "loosening up" of the ribosome structure. The extent of this must be limited since the magnitude of both $\Delta S°$ and $\Delta H°$ are not overly large, and the sign of the latter is opposite to that expected. The conformational changes clearly cannot encompass the melting out of extensive regions of the double-stranded portion of the ribosomal ribonucleic acid (RNA) structure, or comprise the complete denaturation or unfolding, with concomitant solvation, of one or more of the ribosomal proteins. For either of these possibilities, or a combination of the two, limited changes are clearly conceivable. It is also unlikely from the positive value of $\Delta S°$ that the binding mechanism involves a hydrophobic burying of DSM within the ribosomal structure; such a mechanism would require a reasonably large negative entropic change in view of the highly hydrophilic character of the streptomycin antibiotics.

More direct evidence for a conformational change has been obtained by other approaches. Sherman and Simpson (14) reported differences in the exchangeable hydrogens on the ribosome induced by SM in the susceptible, but not the resistant, phenotype. The exact mechanistic interpretation of their findings is not clear at present and differs somewhat from the interpretations of the present binding study. Their interpretations are further complicated by the fact that salt-washed ribosomes were used at low temperatures, and, since thermal activation is negligible under these conditions, the binding activity must be quite low as demonstrated here and by Vogel et al. (16). The thermal activation was not known at the time of the Sherman and Simpson study, and this important investigation needs repetition under conditions where the binding activity of the ribosomes is quantitative or nearly so. More recently in this laboratory, Zitomer (*unpublished data*) has found both that there are more exposed sulfhydryl groups and that the rate of their reactivity to sulfhydryl reagents is greater in the 70S-DSM complex than is the case with the native 70S ribosome.

There are some further indications of the nature of the binding which can be inferred from the specificity and *p*H profile studies of the preceding paper (2). The intact trisaccharide structure of the SM molecule is required, and, since bluensomycin, which lacks one of the two guanido groups of DSM, shows almost equal activity to DSM, it can be inferred that only one guanido group and the secondary amine, in a particular spatial configuration, are required for activity. This configuration is present in the SM antibiotics but absent in the other aminoglycosides. The *p*H profile for binding reveals a range of optimal binding between *p*H 7.4 and 8.2, with a decrease in binding below *p*H 7.4 indicative of the titration of a group with a pK in the vicinity of 6.8. This titratable group must be on the ribosome, because such a group is not present in the SM structure. There is a suggestion from the pK of 6.8 for a secondary phosphoryl group, in which case one of the terminal phosphates of the ribosomal RNA or the possibility of a phosphoryl group on one or more of the ribosomal proteins must be considered. Nevertheless, the energetics of the binding equilibrium suggest that more is involved than a simple electrostatic binding through two of the cationic groups of the SM antibiotics.

With the reversible transition between active and inactive 30S and 70S particles, the argument for a conformational change in ribosome structure is more evident from the thermal requirement for activation, and the structural change here must be more extensive. It is unknown at present whether, during the activation of the reassociated 70S ribosome, the 30S subunit must first be dissociated for activation to occur. There is evidence from the studies of Pestka (13) that the subunit affinity is weaker in reassociated 70S ribosomes than in the native particle, but this could possibly be due to other factors which are removed from native particles in the course of purification. Other thermal activation phenomena involving the ribosome which bear some similarity to the

present findings are the reported activation of the peptidyl transferase activity of the 50*S* subunit (12) and an activation of 70*S* ribosomes (11) and 30*S* subunits (4) for the polynucleotide-stimulated binding of various aminoacyl-transfer RNAs. We have also found that 30*S* subunits inactive for DSM binding are incapable of association with the 50*S* subunit (Zitomer and Flaks, *in preparation*). A single inactivating, conformational change may underlie all of these 30*S* subunit functions, and may also influence other 30*S* functions such as the binding of FMet-transfer RNA and one or more of the initiation factors. It can also be suspected, but the point has not as yet been systematically studied, that the treatment of 70*S* ribosomes with high monovalent salt concentrations in the usual preparative procedures will cause dissociation to 30*S* and 50*S* subunits with substantial inactivation of the 30*S* subunits. This will be true in the presence of 10 mM or higher concentrations of Mg^{2+}. It has been shown that the separation of density-labeled ribosomes by sedimentation in concentrated CsCl, even in the presence of 40 mM Mg^{2+}, yields free subunits (1), and it is known that the presence of increased concentrations of monovalent salts shifts the equilibrium of the Mg^{2+}-dependent subunit association reaction in the direction of increased dissociation (17; Zitomer and Flaks, J. Mol. Biol., *in press*). It therefore seems quite probable that the 70*S* ribosomes that result from any of the purification procedures employing high salt are closely related to, if not identical with, reassociated 70*S* ribosomes.

In Fig. 9 is a summary scheme of the reactions presented in this and the preceding paper (2). The native 70*S* ribosome that is fully active in binding DSM is indicated in the center as $70S_A$, and the four reactions forming the square to the right represent the simplest mechanism that can be proposed for the tight binding of the antibiotic to the 70*S* particle. There is presumed to be an initial collision, possibly diffusion-controlled, between DSM and $70S_A$ to generate the initial collision complex, $70S_A$-DSM, which is then converted at a slower rate to the bound complex, $70S_B$-DSM. The equilibrium lies far in the direction of binding by virtue of the large difference in the two kinetic constants k_1 and k_{-1} but, more importantly, we presume that there is a conformational change in the conversion of the initial collision complex to $70S_B$-DSM. To complete the thermodynamic picture, there is presumed to exist in very minor amounts the ribosome configuration represented by $70S_B$, whose conformation is similar to that existing in the bound complex, $70S_B$-DSM. This same set of reactions can be written for the binding of DSM to the active, free 30*S* subunit, indicated in the lower left corner as $30S_A$, but this has been omitted in Fig. 9 for the sake of clarity in presentation.

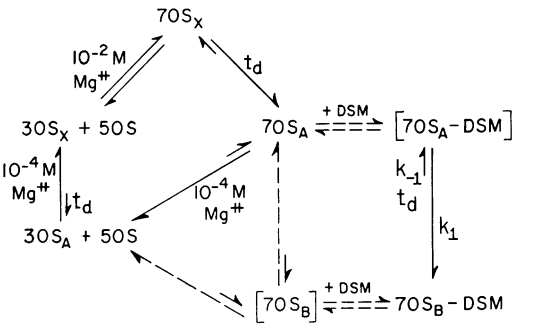

FIG. 9. *Summary scheme for reactions involved in dihydrostreptomycin binding. The terminology for the various reactants is indicated in the text. The term t_d designates a reaction with marked temperature dependence. There is no direct evidence for the bracketed intermediates or the hyphenated reactions; they are presumed to occur on theoretical grounds.*

The reactions to the left in Fig. 9 represent the active-inactive transition of 30*S* subunits and 70*S* ribosomes studied in the present paper and first indicated by Vogel et al. (16), with the active forms indicated by S_A and the inactive ones by S_X. The major difference indicated by the various equilibria is the ability to accumulate large amounts of active 70*S* particles ($70S_A$) in contrast to the active 30*S* subunit ($30S_A$), owing to the instability of the free 30*S* subunit in solution and the stabilization conferred on it when in the 70*S* particle conformation.

The scheme presented here is the simplest one which accounts for the facts known to date, and undoubtedly both the conformational transitions involved in DSM binding and the 30*S* subunit activation are more complex than indicated. There are some indications for this. The biphasic kinetics for both binding and debinding of DSM reported here suggest a heterogeneity for the ribosome not previously indicated. However, despite the biphasic kinetics, all of the antibiotic molecules bound exhibit the same ribosomal affinity. An identical heterogeneity has recently been found for the kinetics of the $30S_A$ to $30S_X$ reaction, and the kinetics of the $30S_X$ thermal activation are rather complex (Zitomer and Flaks, *in preparation*). The simplest explanation at present for these kinetic responses is greater complexity in the $S_A \rightleftarrows S_X$ transition than that indicated in Fig. 9. As indicated in the preceding paper (2), they are not related to the known

compositional heterogeneity of the 30*S* subunit (9, 15).

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