Binding of Isoleucyl Transfer Ribonucleic Acid by Isoleucyl Transfer Ribonucleic Acid Synthetase: Solvents, the Strength of Interaction, and a Proposed Source of Specificity†

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ABSTRACT: The association constant of $tRNA^{Ile}$ (*Escherichia coli*) and isoleucyl-tRNA synthetase has been measured in many solvents varying in ionic strength and composition, and also in solvents which differ by addition of small amounts of organic solvents. It is found that there is a considerable (at least *ca.* tenfold) increase in the affinity of the pair in, *e.g.*, 10% dioxane, as well as an optimum concentration for the strength of binding. Progressive large decreases in affinity are found on addition of monovalent cations, but a more rapid decrease when divalent cations are added. The existence of an optimal concentration of divalent cations can be shown. Analysis suggests that these solvent effects are not simple effects on electrostatic interactions. In addition, highly correlated with the change in affinity, Ile-$tRNA^{Ile}$ goes through a dramatic change in conformation as evidenced by $s^0_{20,w}$. However, this change causes only slight hyperchromicity and no perceptible change in the number of slowly exchanging H atoms. The relaxation of conformation, called "loosening," seems the best explanation for the increased avidity of binding, and the data on all solvents can be interpreted as implying the existence of a conformational state which is optimal for binding. I suggest that loosening speeds the accomodation of tRNA to the protein's surface. Use is made of this concept to outline a mechanism for the recognition reaction (binding) and to discuss the sources of the free energy of interaction. It is suggested that the specificity of interaction between $tRNA^{Ile}$ and its cognate synthetase is attributable, in part, to the occurrence of a compact structure for $tRNA^{Ile}$ in normal solvents.

Perturbation of a system of combining ligands by small changes in solvent is a useful method of analyzing their interaction. I have carried out such a systematic study on the couple Ile-$tRNA^{Ile}$ (*Escherichia coli*)–isoleucyl-tRNA synthetase because of my interest in the source of the specificity of this interaction, whose selectivity is probably required for the preservation of the genetic code. The large changes observed in different solvents suggest that the conformation of the tRNA, and/or the facility with which it can change, are important and thus far undetected contributors to the strength and thereby, probably to the specificity, of this interaction.

Materials and Methods

Isoleucyl-tRNA synthetase is the pure, homogeneous protein from *E. coli* B whose preparation has been previously described (Baldwin and Berg, 1966). The expression $tRNA^{Ile}$ refers to tRNA purified by methods previously discussed (Yarus and Berg, 1969) from *E. coli* B. It is >80% pure, as judged by a comparison of absorbance to acceptor capacity. Unresolved tRNA (*E. coli*), such as that used in certain centrifugation experiments in this paper, is prepared as previously described (Zubay, 1962), then passed over benzoylated DEAE (Gillam *et al.*, 1967) in 1 M NaCl and 0.01 M $MgCl_2$ to remove fragments of DNA and of larger RNAs which contaminate ordinary preparations.

The tRNA binding assay has been described (Yarus and Berg, 1967, 1970). It depends, briefly, on the retention of Ile-$tRNA^{Ile}$ by nitrocellulose filter when it is bound to isoleucyl-tRNA synthetase, and is ordinarily performed in a reaction mixture containing 0.05 M potassium phosphate, 0.010 M $MgCl_2$, 0.0001 M dithiothreitol, 0.0001 M EDTA, and 50 μg/ml of carboxymethylated bovine serum albumin (pH 5.5). When additions were made to these standard reaction mixtures (salts or organic solvents), preadjustment of the pH was sometimes necessary so that the pH of the final mixture would remain at 5.5. Readings obtained with a Corning No. 476050 pH electrode, standardized against phosphate buffer (pH 7.0), were taken at face value.

Measurements of aminoacylation velocity and ATP–PP exchange velocity were carried out in binding reaction mix at 17 or 20°. In the first case, in addition to the basic components of this solution, 0.25–0.40 nmole of [^{14}C]isoleucine (50 Ci/mole), 0.4 μmole of ATP, and 1 μmole of L-Ile were added to a final volume of 1 ml of standard binding reaction mixture (Yarus and Berg, 1970).

Measurement of the velocity of Ile-tRNA release from isoleucyl-tRNA synthetase was carried out by allowing 44 pmoles of isoleucyl-tRNA synthetase to come to equilibrium with 87 pmoles of [^{14}C]Ile-$tRNA^{Ile}$ (312 Ci/mole) in 1 ml of standard binding reaction mix (±5% dioxane by volume, ±1 μmole of L-Ile). Then 300 pmoles of $tRNA^{Ile}$ was added to begin the experiment and aliquots were taken at intervals for assay.

When required, the molar concentration of enzyme was measured by titration with ATP and [^{14}C]Ile (Yarus and Berg, 1970). Centrifugation was performed in a Spinco Model E ultracentrifuge equipped with an ultraviolet scanner. Cell assemblies consisted of quartz windows and 12-mm charcoal filled-epon double-sector centerpieces were used. The speed for velocity sedimentation experiments were usually 48 krpm, and 0.10–0.20 A_{260} of Ile-$tRNA^{Ile}$ used; the midposition of the boundary was followed to determine s^0. $s^0_{20,w}$ was determined by correction of the observed s^0 using values for the viscosity

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and density of mixed solvents from Timmermans (1960), which was also the source of the dielectric constants. Usually several experiments were done at once in a 4-place rotor (Spinco ANF).

The equilibrium centrifugation referred to in the text was performed using a starting concentration of 0.15 A_{260} of Ile-tRNA^{Ile}. A 3-mm column was centrifuged at 32 krpm and 17° to deplete the meniscus (Yphantis, 1964). Control experiments showed that Ile-tRNA survives centrifugation.

Tritium-exchange measurements were made by the "one-column technique" of Englander (Englander, 1968), using jacketed 0.8 × 8–9 cm columns of Sephadex G-25 fine, which were maintained at 0° by circulating H_2O–methanol in the jackets, and eluted with binding reaction solution (without albumin). [^{14}C]Ile-tRNA was equilibrated in HTO (New England Nuclear) of final specific activity 100 mCi/ml for 5 min at 37°. Then a premixed binding cocktail was added (in 0.3 volume) to give final conditions equilvaent to a binding reaction mixture, and incubation was continued for 25 min at 25°. The tRNA was then iced and filtered in aliquots over the jacketed columns. Only about 0.04 A_{260} of [^{14}C]Ile-tRNA was used per kinetic point, and the number of ^{3}H bound per tRNA was calculated from the ratio of ^{3}H:^{14}C in the tRNA peak. When dioxane was present, it was a constituent of the column eluent only.

This preequilibration procedure gave maximal ^{3}H bound; longer preincubation does not give more. Various lower specific activities of tritiated water down to 0.05 that customarily used gave figures comparable to that obtained with the rather high specific activity usually used.

Hyperchromicity measurements were made by volumetric addition of reagents to a solution of Ile-tRNA^{Ile} held in the thermostated (17°) sample chamber of a Gilford Model 2000 spectrophotometer.

Results

Large, unmistakable changes occur in the affinity of Ile-tRNA for isoleucyl-tRNA synthetase when moderate changes are made in the millieu; some binding data for normal conditions, increased ionic strength, and normal ionic conditions plus organic solvent are compared in Figure 1. As is also shown there, these changes in the association constant occur without concurrent changes in the number of sites for binding Ile-tRNA; that is, the extrapolates of the various double-reciprocal binding curves are similar and all close to 1 mole of tRNA/mole of isoleucyl-tRNA synthetase.

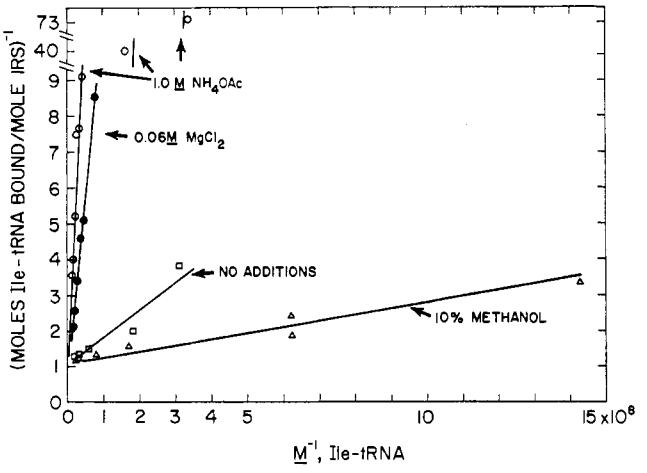

FIGURE 1: Binding of [^{14}C]Ile-tRNA to isoleucyl-tRNA synthetase double-reciprocal plots. In this figure, the extrapolate at the left is reciprocal number of sites for tRNA/mole of isoleucyl-tRNA synthetase, and the slope of the lines is the reciprocal of the association constant. The lettering by the lines indicates what, if any, additions were made to the standard binding reaction. IRS is used, in this figure, as an abbreviation for isoleucyl-tRNA synthetase.

In Figure 2A,B a larger amount of such data is summarized. Here each binding curve like those in Figure 1 is represented only by one point. The ordinate is the log of the association constant, k (the reciprocal of the dissociation constant, K, usually considered in enzyme kinetics), derived from the slope of the double-reciprocal plot, and the abscissa has been selected to permit a comparison to the predictions of electrostatic theory. Figure 2A shows the effect of log k (which is a convenient quantity because it is proportional to the strength, or more precisely, the standard free energy of interaction, $\Delta G°$) when the ionic strength is increased by adding monovalent or divalent cations. In both cases the interaction rapidly weakens. If weakening were due to the expected effect of added salt on an electrostatic interaction, then the decline in log k should[1] be linear in some function of $(\mu)^{1/2}$ (where μ is the ionic strength). Consideration of the data in Figure 2A shows that no such plot will give straight lines for the data for both the monovalent and divalent cations. The behavior is far more complex. Because of the approximate nature of the theory, this failure is ambiguous, though a similar analysis of data (Latt and Sober, 1967) on the poly(I + C)–oligolysine interaction, which is believed to be electrostatic, does give linear or near-linear plots.[2]

The differences between curves are less ambiguous. The behavior on addition of divalent cations is quite different from monovalent ions: I stress that this cannot be reconciled with *any* explanation based solely on electrostatics, and suggest that the correct explanation must take account of the "chemical" differences between these ions. Because of the well known (Nishimura and Novelli, 1963) stabilization of compact structures in nucleic acids by Mg^{2+}, I suggest that progressive stabilization of compact form(s) of tRNA results in a rapid decline in affinity. This impression is reinforced by a third characteristic of the system; the indication (in the Mg^{2+} data, see inset in Figure 2A) that there is an *optimal* Mg^{2+} concentration, considerably lower than the concentration of Mg^{2+} in the standard assay. That is, the association constant is considerably greater in 5 × 10^{-4} M Mg^{2+} than in either the absence of added Mg^{2+} or at 10^{-2} M Mg^{2+} (inset, Figure 2A). Thus, under standard binding conditions at 17° and 10^{-2} M $MgCl_2$, the secondary and tertiary structure of Ile-tRNA is more than optimally stable, and it is expected that addition

[1] A very approximate treatment of ionic interactions based on the Debye-Hückel theory for two spherical macroions of equal radius much larger than their counterions gives

$$\log k = C + \frac{A}{D}\frac{1}{1 + B(\mu/D)^{1/2}}$$

A, B, and C are constants (the last represents that part of the interaction which is unaffected by salt, μ is ionic strength, and D is dielectric constant. Use of realistic values in this equation suggests that log k should be linearly related to $1/(1 + 3.25\mu^{1/2})$ when ionic strength is varied at constant dielectric constant (that of H_2O at 20°); and linearly related to $1/(D + 7.7D^{1/2})$ when ionic strength is held constant and D varied. While this rather specific theory is used in the text, it should be clear that the argument does not depend on it, and plotting *vs.* any function of salt concentration would do as well.

[2] M. Yarus, unpublished data.

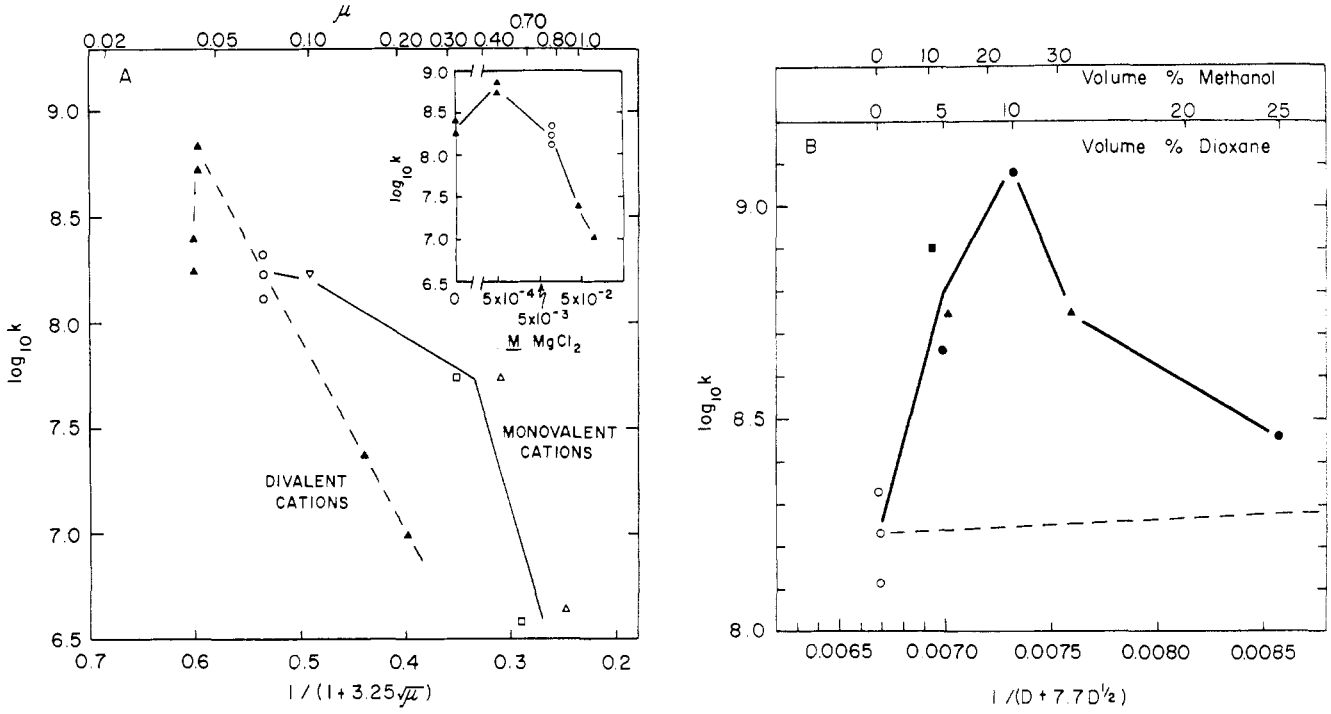

FIGURE 2: (A) The effect of variations in ionic strength on association of Ile-tRNA and isoleucyl tRNA synthetase. In the major figure the logarithm of the association constant is plotted *vs.* an appropriate[1] function of the ionic strength, μ. The solid line describes the behavior when monovalent ions are added: ▽, KSCN; □, KCl; △, NH_4OAc. Open circles are data for standard binding conditions. The dashed line describes the behavior when the concentration of divalent cations (▲, $MgCl_2$) is varied. The concentration of Mg^{2+} under standard binding conditions is 0.01 M. In the inset, the data for log k, which is proportional to ΔG°, the standard free energy of binding, is replotted as a function of the Mg^{2+} concentration to emphasize the existence of an optimum. (B) The effect of addition of organic solvents on association of Ile-tRNA and isoleucyl-tRNA synthetase. The logarithm of the association constant is plotted[1] *vs.* $1/(D + 7.7D)$, in which D is the bulk dielectric constant. On the upper scale, the corresponding volume per cent dioxane is indicated: ○, normal binding conditions; ●, dioxane added; ▲, ethanol added; ■, methanol added. The dashed line approximates the behavior expected if the changes in solvent were acting on an electrostatic interaction involving unit charges. The slope of this latter line should be approximately proportional to the product of the charges involved.

of monovalent ions will lead to a decline in affinity, as is observed.

To summarize, then, while isoleucyl-tRNA synthetase may perceive the charge distribution of Ile-tRNA (In fact, we have evidence from the study of competitors for isoleucyl-tRNA synthetase which indicates that it does do so to some extent.[2]), the nature of the effects of ionic strength suggests a large indirect effect of ions through their effect on conformation of tRNA.

The strong binding of $tRNA^{Ile}$ in the absence of any added divalent ion (inset of Figure 2A) is of incidental interest; we have shown elsewhere under conditions in which divalent ion concentrations are rigorously controlled that $tRNA^{Ile}$ does not require a divalent ion either stoichiometrically or catalytically in order to be recognized and aminoacylated (Yarus and Rashbaum, 1972). Thus, the effect of monovalent ions cannot be attributed to, *e.g.*, displacement of a required multivalent cation.

If Ile-tRNA is too stable for optimal binding, then a reagent or condition which loosened the structure present under binding conditions in the proper way will increase affinity. It is in the light of an optimal conformation or an optimal mobility of conformation that we wish to view the data on Figure 2B, in, which are shown the effects of addition of methanol, ethanol, or dioxane to the standard binding reaction mixture. The abscissa is a function of D, the reciprocal dielectric constant, because it would be expected[1] that log k or ΔG° would vary as $1/(D + 7.7\ D^{1/2})$ for an electrostatic interaction. For comparison, the dashed line originating at the leftmost (H_2O only) point in Figure 2B portrays the approximate behavior expected for an attractive electrostatic pairing involved unit charges. The effects observed are clearly too complex (again showing an optimum)[3] to be explained as a simple dielectric effect on a fundamentally electrostatic interaction. As we will

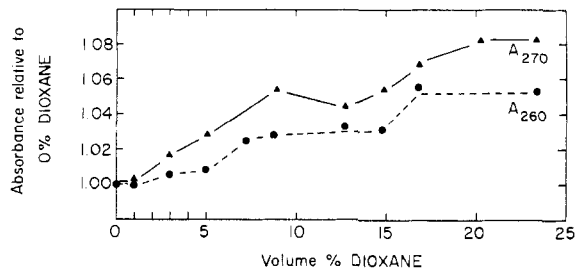

FIGURE 3: Hyperchromicity of Ile-tRNA on addition of dioxane to standard binding conditions. Albumin has been omitted from these samples.

[3] The increase in association constant shown in Figure 2 is almost a factor of 10. However, the actual change could be larger. As the binding constant approaches and exceeds 10^9 l./mole, the concentration of free Ile-tRNA becomes so small under standard binding conditions (Yarus and Berg, 1970) that it is difficult to measure convincingly. Thus I do not wish to stress the absolute value of the maximum k in Figure 2, nor should arguments be made in which its exact value is a critical element.

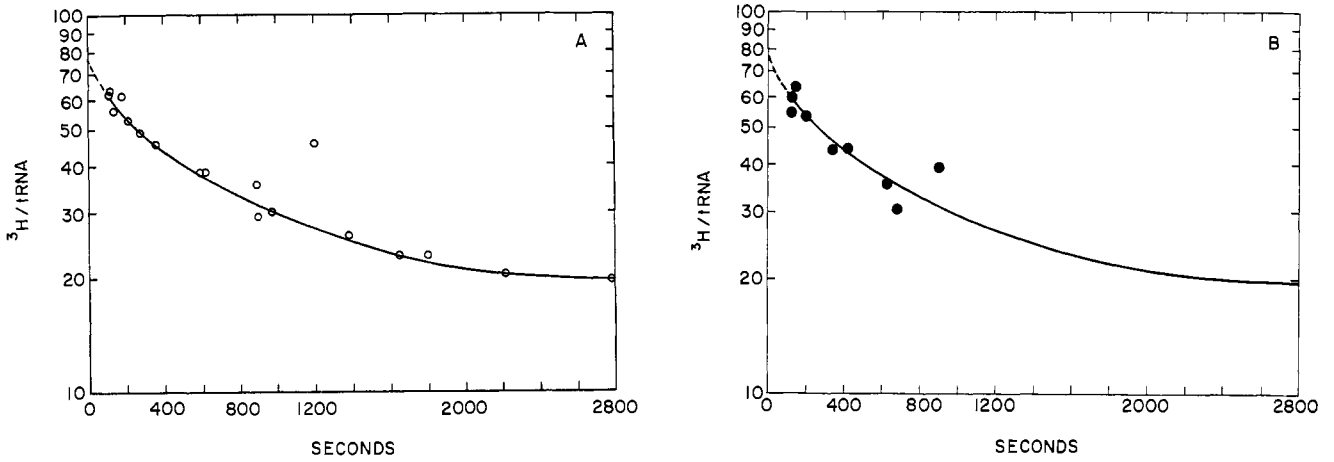

FIGURE 4: Tritium exchange in Ile-tRNA. (A) The exchange of hydrogens from Ile-tRNA at 0° in standard binding conditions. (B) The exchange of hydrogens from Ile-tRNA at 0° in standard binding conditions plus 10% dioxane (filled circles). The line represents normal behavior, repeated from Figure 4A.

see below, there is a conformational change in tRNA under these conditions.

The organic solvents used here are denaturants for nucleic acids; thus the data in Figure 2B could be plausibly explained as a result of change in tRNA secondary or tertiary structure or in its stability. However, only small amounts of organic solvent and low temperatures (17°) have been used in this study. By comparison with the much more rigorous conditions required to disorder double helices, the change provoked by these conditions should be quite subtle. This is, in fact, the case, as shown in Figure 3, which plots the hyperchromic effect *vs.* amount of organic solvent added. As can be seen, the net amount of short to moderate-range order of the bases in Ile-tRNA is affected only slightly by the addition of, *e.g.*, 10% dioxane (~3% hyperchromicity at 260 nm; Figure 3).

One has the impression, on looking at hyperchromicity data, of a molecular change which is complex, having a first stage in which there is a progressive change between 0 and 8–10% dioxane, then a plateau or, in some data, a decrease in absorbance. At 13–15% dioxane, another change involving net disordering begins. As we shall see below, this picture is consistent with measurements of $s^0_{20,w}$. No hyperchromicity is found when dioxane is added to an equimolar mixture of nucleotides, so that it is clear that these are effects mediated by secondary and tertiary structure.

Similar data for the addition of salts to normal binding conditions (not shown) suggest little, if any, effect of these additions on the absorbance of Ile-tRNA.

The net number and kinetics of exchange of hydrogens on groups involved in secondary and tertiary structure (Englander 1968; Hanson, 1971) in standard binding conditions, as measured by the number of slowly exchanging ^{3}H in Ile-tRNA equilibrated with $^1HO^3H$, is shown in Figure 4A. It may be seen that purified Ile-tRNA has ^{3}H-exchange kinetics similar to the samples of mixed tRNAs which have been previously tested (Englander and Englander, 1965). Extrapolation of the specific activity of ^{3}H to zero time of exchange suggests about 75 slowly exchanging hydrogens Ile-tRNA, and there are several stability classes among hydrogens, the more slowly exchanging being revealed after the faster exchanging ones have been released.

The extrapolated value is less than the 88–95 (Gantt *et al.*, 1969) hydrogens involved in base pairing which have been found for mixed *E. coli* tRNAs, but the apparent number of slowly exchanging hydrogens is reasonably sensitive to ionic and other conditions. In fact, measurements on mixed, unacylated *E. coli* tRNAs in these conditions suggest that the average tRNA has a similar extrapolate, but a faster rate of exchange. Thus the slowly exchanging hydrogens of Ile-tRNA^{Ile} are less accessible to solvent than are the hydrogens of an average unacylated *E. coli* tRNA.

As can be seen in Figure 4B, the total number of detectable hydrogens and the early kinetics of exchange are little affected by the presence of 10% dioxane in the medium during exchange. There is clearly room in the variation of the data for a difference of several hydrogens, but large changes are ruled out. I conclude that 10% dioxane added to standard binding conditions disrupts fewer than 5% of the H bonds in the native Ile-tRNA molecule.

On the other hand, there is a marked change in the shape of the molecule under these conditions which can be followed by measuring $s^0_{20,w}$. This is shown in the several panels of

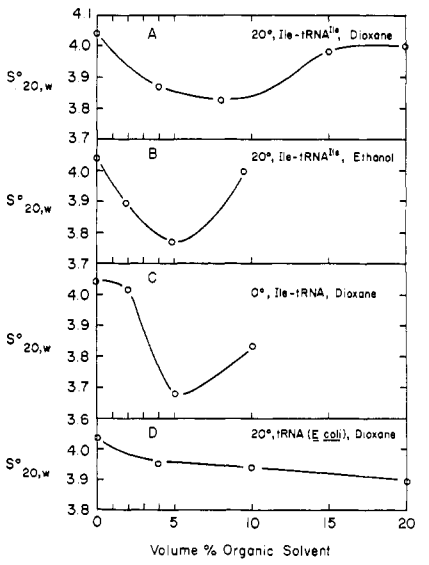

FIGURE 5: The effect of additions of organic solvent to standard binding conditions on the corrected sedimentation coefficient of Ile-tRNA. (A–C) Ile-tRNA, various solvents, and temperatures; (D) mixed acceptor from *E. coli* B.

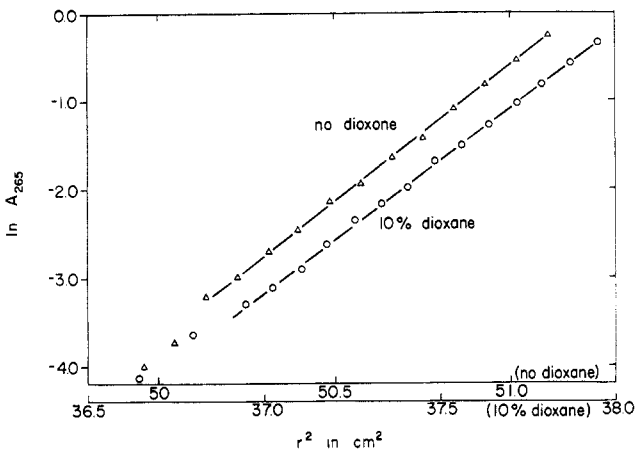

FIGURE 6: Equilibrium sedimentation of Ile-tRNA with and without 10% dioxane. Meniscus depletion methods (Yphantis, 1964) were used and the absorbance profile evaluated from photoelectric scanner records after sufficient time (22 hr at 17° and 32 krpm) so the concentration distribution had stopped changing, as judged from successive records several hours apart.

Figure 5. For several reasons, these measurements were made at very low concentrations of Ile-tRNA, 0.1–0.2 A_{265}/ml. This is far below the range of concentrations at which concentration dependence of sedimentation is observable (Henley *et al.*, 1966) and so observed values are equivalent to s^0, the sedimentation constant at zero concentration, and need not be corrected for concentration. In addition, these concentrations are only slightly greater (~2-fold) than the higher concentrations of Ile-tRNA used in collecting binding curves, thus simulating those conditions closely. Lastly, aggregation of tRNAs, observed at much higher concentrations (Millar and Steiner, 1966), should not be significant at these low concentrations (see immediately below).

Thus, as seen in Figure 5, Ile-tRNA at 20°, or at 0°, undergoes a drop in $s^0_{20,w}$, and then a rise again, as ethanol, or dioxane is progressively added to the solvent. This change in the frictional coefficient[4] occurs in the same range of conditions under which the affinity increases on addition of organic solvent (*cf.* Figures 2B and 5). It is difficult to draw an exact parallel because the complex changes in the shape of the tRNA molecule probably include variations which have little effect on binding.

I conclude that addition of these solvents causes changes in the frictional coefficient of Ile·tRNA under the same conditions in which affinity for isoleucyl-tRNA synthetase is enhanced. Because the effect of organic solvents at the lowest concentrations is clearly in the direction of expansion of the tRNA molecule and enhanced binding and because of the analogy to the data on ions (Figure 2A), these data suggest that an overall relaxation of its compact structure, which I will call "loosening," is correlated with the enhancement of binding. I suggest that it is likely that this is also the major cause of the greater binding free energy. The increase in $s^0_{20,w}$ is tentatively interpreted as collapse of the molecule, and the fall of the association constant is regarded as indicating that, at higher concentrations of organic solvent, a second effect predominates over denaturation. These organic solvents, if added to sufficiently high concentrations are precipitants; in other words, they are "poor" solvents in which nucleic acids will prefer to associate rather than be in solution. I surmise that the supposed collapse of the expanded tRNA could be an early expression of this latter effect.

Panel C of Figure 5 shows that the change in s occurs under the conditions in which tritium exchange was measured. The panel which shows data for unacylated, unresolved tRNA (*E. coli*) (Figure 5D) suggests the average *E. coli* tRNA species is characterized by a decrease in $s^0_{20,w}$ at low concentrations of organic solvent, though a smaller one than $tRNA^{Ile}$. Sedimentation patterns do not become noticeably heterogeneous under these conditions, which suggests that most tRNAs are involved, though the expected differences are small, and a more quantitative conclusion would require extensive analysis. Below (in the Discussion), I will return to the question of the generality of this effect. It seems, however, that other species of tRNA (*E. coli*) are, on the average, less sensitive to the effect of higher concentrations of organic solvent than is $tRNA^{Ile}$ (*E. coli*); the $s^0_{20,w}$ does not increase again, even at 20 vol % dioxane, as it does at the higher concentrations in the top three panels of Figure 5.

It is clear that changes in corrected sedimentation velocity of the order reported here (5–10%) indicate very large changes in molecular structure, because of the insensitivity of the frictional coefficient to most types of change: for example, the larger change would be encountered in increasing the axial ratio of an oblate elipsoid from 4:1 to 7:1, a change that would clearly require extensive, molecule-wide rearrangement. I stress that this example is *not* given to suggest that organic solvents dramatically elongate tRNA, but only to indicate that we are dealing with an extensive change in shape. I will discuss this subject further below.

Figure 6 shows the distribution of Ile-tRNA after centrifugation to equilibrium under standard binding conditions (albumin omitted) and with 10% dioxane added to these conditions. The slope of these plots is proportional to molecular weight and to $(1 - \bar{v}\rho)$. The agreement between the slopes is at least partially an accident resulting from a cancellation of effects: the $\bar{v}$ of tRNA probably decreases slightly in 10% dioxane (see footnote 1), but the density of the solvent increases. In any case, the effects are small and it is clear that there is no large change in molecular weight on addition of 10% dioxane. In addition, the method of meniscus depletion ensures that a wide variety of concentrations will be present in the cell at equilibrium. From the point at the left in Figure 6, where deflections (absorbance) become large enough to be reliable (from $A_{265} \sim 0.04$), to the bottom of the cell, a range of about 20-fold in tRNA concentration is covered in Figure 6. Over this entire range the data are a good fit to a straight line: the absence of upward curvature, which would indicate aggregation, is an excellent indication that Ile-tRNA exists as an unaggregated monomer under the conditions of these experiments. The slope (in 0% dioxane) corresponds to a molecular weight of 27,200 ($\bar{v} = 0.53$), in excellent agreement with the result (26,600) expected from the sequence of $tRNA^{Ile}$ (Yarus and Barrell, 1971).

In Figure 7 are data on $s^0_{20,w}$ covering the range of salt concentrations shown to have strong effects on affinity in Figure 2. No systematic effect of addition of high salt or Mg^{2+} is

[4] In principle, changes in molecular weight, partial specific volume ($\bar{v}$), or frictional coefficient could be responsible for a change in $s^0_{20,w}$. However, molecular weight changes have been ruled out by equilibrium sedimentation under these conditions (Figure 6). Reducing water activity by addition of organic solvents should decrease $\bar{v}$, which would increase, not decrease, sedimentation coefficient. This last is, in fact, the way the apparent $\bar{v}$ behaves[2] in a determination by sedimentation in H_2O–D_2O–dioxane solutions (Edelstein and Schachman, 1967). Thus we are left with the increase of the frictional coefficient as an explanation for the decrease in $s^0_{20,w}$ seen here.

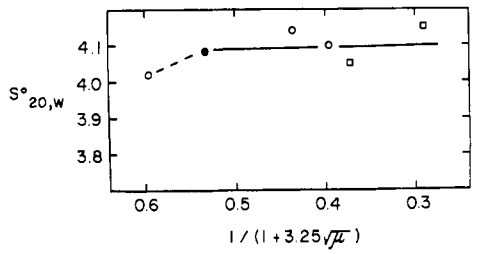

FIGURE 7: The effect of salt concentration on the sedimentation of Ile-tRNA. (●) Standard binding conditions; (○) $MgCl_2$ added to a final concentration of 0.04 and 0.06 M left to right; (□) KCl added to a final concentration of 0.20 and 0.50 M; $MgCl_2$ reduced to 5×10^{-4} M.

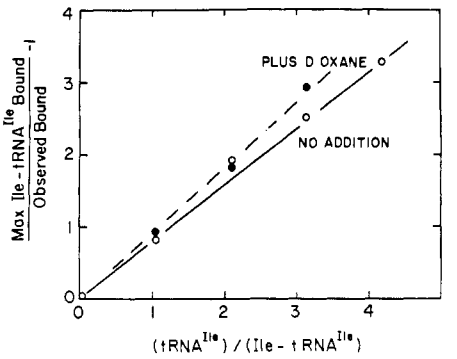

FIGURE 8: Competition between Ile-tRNA^{Ile} and tRNA^{Ile} for isoleucyl-tRNA synthetase in the presence and absence of 10 vol %, dioxane. As the effectiveness of tRNA^{Ile} as a competitor increases, the slope of the line will increase (Yarus and Berg, 1967).

seen, implying that neither large changes in structure nor aggregation of Ile-tRNA is occurring in these experiments. There may, however, be a decrease in $s^0_{20,w}$ on reduction of (Mg^{2+}) from 0.01 to 0.0005 M.

The smaller magnitude of the conformational effects here (Figure 6), which are nevertheless accompanied by an increase in affinity for isoleucyl-tRNA synthetase (*cf.* Figure 2A) probably indicates that the change in conformation evoked by organic solvent is complex. Perhaps only a small part of the changes which occur directly affect the affinity. To put this point about the relation between overall changes and the changes relevant to the affinity in its extreme form, these data show that changes which destabilize the compact conformation (addition of organic solvent, or lowering Mg^{2+}) increase the affinity. Though this be true, there need not be *any* essential change in the overall conformation of tRNA in solution; it could alternatively be that the essential aspect of these changes in solvent is to increase the ability of the structure to change as its conforms to the surface of isoleucyl-tRNA synthetase. While the changes in tRNA structure which do occur (Figure 5) are unlikely to be irrelevant to the progress of the tRNA onto the enzyme's surface, they need to be used in the argument of the paper only to show that the solvent does, as expected, have conformational effects. One would be surprised, in fact, to find that such a large change was correlated in every respect with the increase in affinity. This, indeed, is the rationale for the selection of the word, loosening, to represent the solvent effect: it does not imply a choice between the alternatives; that the essential effects of solvent are on conformational change directly, or only on the potential for change.

We now turn to the question of whether solvent loosening, which has so far been characterized using [^{14}C]Ile-tRNA^{Ile}, also affects tRNA^{Ile}, the unacylated tRNA. An experiment in which [^{14}C]Ile-tRNA and unacylated, purified tRNA^{Ile} compete for binding to a limited amount of isoleucyl-tRNA synthetase (±10% dioxane) is shown in Figure 8. The data are plotted so that the slope of the straight line obtained will be the ratio of the association constant of tRNA^{Ile} to that of Ile-tRNA^{Ile} (Yarus and Berg, 1967). We know (Figures 1 and 2) that addition of organic solvent will increase the affinity of Ile-tRNA for isoleucyl-tRNA synthetase; if the affinity for tRNA^{Ile} is not also increased, then the slope of the curve determined in the presence of dioxane should be much less than that in its absence. In Figure 8, it can be seen, first of all, that the slope of the curve in the absence of dioxane is about 0.8; this implies, as has been pointed out below (Yarus and Berg, 1967), that the addition of the Ile residue to tRNA^{Ile} adds slightly to the association of the tRNA with Ile-tRNA synthetase; the association constant for tRNA^{Ile} is about 0.8 that for Ile-tRNA^{Ile}. When dioxane is present, competition by tRNA^{Ile} is reproducibly stimulated slightly (Figure 8). Thus the binding of tRNA^{Ile} to isoleucyl-tRNA synthetase is also stimulated by organic solvents, and in fact, may be slightly more so than Ile-tRNA^{Ile}. I conclude that this effect, then, is not a pecularity of Ile-tRNA^{Ile}, but is also a property of the unacylated tRNA^{Ile}.

To this point I have, for clarity, talked entirely in terms of the effect of the solvents on tRNA. I now turn to their effects on isoleucyl-tRNA synthetase.

By adding isoleucyl-tRNA synthetase to binding reaction mixtures containing the various organic solvents and salts used in this work, and then diluting to remove these agents at successive times, it can easily be shown that little if any irreversible change in the enzymatic activity of Ile-tRNA synthetase occurs during the brief (20- to 30-sec) period of exposure required to do a tRNA binding assay (Yarus and Berg, 1969). The values in Table I are representative, though it has proven difficult to reproduce inactivation curves in some cases; apparently organic solvents irreproducibly labilize isoleucyl-tRNA synthetase to inactivation during the dilutions necessary to these assays. I conclude, however, that neither that part of isoleucyl-tRNA synthetase concerned with activation of isoleucine nor that for tRNA binding and transaminoacylation are permanently altered during tRNA binding assays by the variety of solvent conditions used in this work. This conclusion is strongly confirmed by the observation of a relatively constant total number of tRNA binding sites in these experiments, indicating that the tRNA site is not damaged by these variations in solvent (see Figure 1 for some data).

A much more elusive possibility remains: that alteration of the solvent reversibly alters the conformation of isoleucyl-tRNA synthetase, and among these alterations is one which has a large effect on the region of the tRNA site and thereby on its affinity for tRNA.

This possibility, however, seems unlikely on general grounds; physical techniques suggest that protein structure is usually unaffected by methanol, ethanol, or dioxane to at least 20% volume (Tanford, 1968), and the affinity for tRNA^{Ile} is already strongly (and similarly) affected by these solvents at 5% or 10% concentration by volume (Figure 2). In fact, it may be found by extrapolation from the data of Schrier *et al.* (Schrier *et al.*, 1965) that >90% methanol would be required to bring the spectrophotometrically determined denaturation temperature of RNase down to 20° (binding experiments are

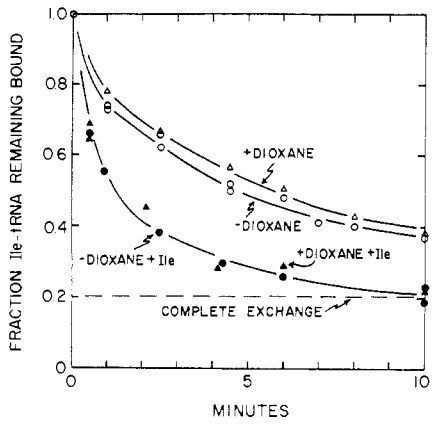

FIGURE 9: Release of Ile-tRNA from isoleucyl-tRNA synthetase in the presence and absence of isoleucine and dioxane. Measured by adding (time zero) an excess of $tRNA^{Ile}$ to a preformed isoleucyl-tRNA synthetase-[^{14}C]Ile-tRNA complex (see Methods).

usually performed at 17°). Muscle ATPase is active up to at least 40 vol % methanol, and 30 vol % dioxane (Laidler and Ethier, 1953), and trypsin is active in 88% dioxane (Inagami and Sturtevant, 1960). Thus the conditions which provoke increased affinity are not expected to have large effects on the structure of most proteins,

However, it is necessary to deal with this protein, under these conditions. This is quite difficult because the hypothetical conformational change could be very subtle. And even given a small change in, say, circular dichroism, it would be difficult to relate it to the effect on binding. Therefore, I have chosen to measure the function of the protein, and in particular, the rate of release of Ile-tRNA from the tRNA site of isoleucyl-tRNA synthetase. This rate should be sensitive to changes in the protein around the tRNA site. It is also conveniently accessible. It may be measured directly or by measuring the overall velocity of aminoacylation, since the rate of aminoacyl transfer is, under these conditions, limited by and similar to the rate of dissociation of Ile-tRNA from isoleucyl-tRNA synthetase (Yarus and Berg, 1969; Eldred and Schimmel, 1972). Thus the experiment to be done consists of a direct or indirect measurement of Ile-tRNA release in the continuous presence of the varied solvent conditions of interest. The success of the experiment requires that solvent effects on tRNA also be irrelevant to the velocity of release. Thus if the effects of solvent modification are small, one concludes that *neither* the solvent effects on the tRNA, nor the hypothetical solvent effects on the protein are important to release.

In Figure 9 is shown kinetic data on release of Ile-tRNA from isoleucyl-tRNA synthetase. These experiments are done by a method described previously (Yarus and Berg, 1969) which involves measurement of the rate at which [^{14}C]Ile-tRNA leaves a preformed complex when an excess of $tRNA^{Ile}$ is added. As seen in Figure 9, release is the same in the presence and absence of 5% dioxane, whether or not L-Ile is present, though L-Ile speeds release, as has been shown before (Yarus and Berg, 1969). Since the association constant is enhanced (Figure 2) in these conditions, but the rate of dissociation is not (Figure 9), it seems that association of $tRNA^{Ile}$ and isoleucyl-tRNA synthetase must be the event whose rate is enhanced by low concentrations of dioxane. The alternatives, that both association and dissociation would be affected, or that organic solvents would slow dissociation only, are eliminated. That is, loosening has been shown to help $tRNA^{Ile}$ to make effective contact with isoleucyl-tRNA synthetase. This conclusion does not depend on the effect on isoleucyl-tRNA synthetase, or lack of it.[5]

This observation, in addition, is also most simply interpreted to mean that the mechanism of Ile-tRNA release is unaltered in dioxane. That is, (a) regaining a more compact structure is not a rate-limiting step in release by the criterion on the filter assay because release occurs at the same rate whether opening is facilitated or not, and (b) the geometry of the tRNA site has not been changed by dioxane.

However, a coincidental and precise cancelling of these latter effects could also account for the lack of change on addition of dioxane. I will now show that over a wide range of conditions, including those in which the apparent affinity of tRNA of isoleucyl-tRNA synthetase is reduced several orders of magnitude instead of enhanced, a similar constancy is observed. This makes it very unlikely that the effect of solvent on Ile-tRNA (which will have been tested under conditions which favor both opening and closing) could be accidentally cancelling the effect of a change in protein conformation.

Rates of aminoacylation have been measured (Table II) with salts and organic solvents continuously present in the assay.

TABLE I: Irreversible Effect of Various Solvents on Isoleucyl-tRNA Synthetase.[a]

Agent	Act. Assayed	Time and Temp (°C) of Exposure	Fraction Act. Surviving
None	Aminoacylation	30 sec at 17	1.0
25% Methanol	Aminoacylation	30 sec at 17	1.0
25% Ethanol	Aminoacylation	30 sec at 17	1.0
25% Dioxane	Aminoacylation	30 sec at 17	0.88
0.2 M KCl	Aminoacylation	30 sec at 17	0.98
1.2 M KCl	Aminoacylation	30 sec at 17	0.95
0.2 M NH_4Cl	Aminoacylation	30 sec at 17	1.0
0.2 M NH_4OAc[b]	Aminoacylation	30 sec at 17	1.0
1.0 M NH_4OAc	Aminoacylation	30 sec at 17	1.04
1.0 M NH_4OAc	ATP–PP exchange	20 sec at 20	0.98
10% Dioxane	ATP–PP exchange	30 sec at 20	0.97
25% Dioxane	ATP–PP exchange	20 sec at 20	0.93
10% Ethanol	ATP–PP exchange	30 sec at 20	0.98

[a] In these experiments, the agent, adjusted to pH 5.5, was added to binding reaction solution, and then enzyme was added and then removed at various times for dilution and assay. Surviving fraction of activity is a derived figure, gotten by using the inactivation curve so determined. The inactivation at the time usually required for a binding assay, 20–30 sec, was interpolated from the curves when there was no point at the appropriate time. [b] NH_4OAc is ammonium acetate.

[5] The rate of association is high enough ($k_{forward}$ = several × 10^8 sec^{-1} (Yarus and Berg, 1969)) so that the usual reaction mixtures must be extensively diluted (10^5- to 10^6-fold) to make the rate measurable by the filter assay. But then the overall fraction of the tRNA which will react is so small that accurate measurements cannot be made. This technical difficulty has so far prevented a dependable direct measurement of forward rates.

Under these conditions, the observed activity is reduced by whatever irreversible inactivation is suffered by the enzyme during the assay period. Inactivation which occurs during these long incubations may be corrected by using the body of data which also gave rise to Table I. This has been done in the "corrected activity" column of Table II. Because of the difficulty of reproducing inactivation rates (referred to above), this column is only approximate. For example, this probably accounts for the small difference between the 5% dioxane entry in Table II and the data in Figure 9. Inspection of these data justifies the conclusion that the large effects of solvents on the affinity of isoleucyl-tRNA synthetase for $tRNA^{Ile}$ (Figure 2) are not reflected either in the magnitude or in the complexity of effects on velocity of aminoacylation. Because the rate of aminocyl transfer is normally limited under these conditions by the rate of dissociation of Ile-tRNA from isoleucyl-tRNA synthetase (Yarus and Berg, 1969; Eldred and Schimmel, 1972), rate of release of Ile-tRNA from isoleucyl-tRNA synthetase is little modified by this range of solvents. These data thus strongly suggest that this range of solvents does not modify the vicinity of the tRNA site of isoleucyl-tRNA synthetase in any *functionally* important respect. It also suggests the additional conclusion that the major effect of salts, like organic solvents, is on the association of tRNA and isoleucyl-tRNA synthetase. They reduce affinity (Figure 2), but do not similarly reduce aminoacylation velocity (Table II). Salts, particularly those containing Mg^{2+}, make it more difficult for $tRNA^{Ile}$ to conform to the surface of isoleucyl-tRNA synthetase.

Thus these experiments have not revealed the flexibility of the tRNA site region of isoleucyl-tRNA synthetase which would be required to explain the behavior depicted in Figure 1 either in the presence of salts or in organic solvents. In addition there is a change in $tRNA^{Ile}$ (Figure 5) which may be developed into a unitary, plausible account of all these effects. Therefore, I regard $tRNA^{Ile}$ as the member of the pair whose conformation is significantly affected by solvents.

TABLE II: Aminoacylation[a] of tRNA in the Presence of Salts and Organic Solvents.

Agent[b]	Fraction Act. = Obsd Rate/ Obsd Rate in Absence of Agent	Corr Act.[c]
None	1.0	1.0
5% Dioxane	0.53	0.66
10% Dioxane	0.31	0.64
25% Dioxane	0.048	0.23
5% Methanol	0.68	0.72
10% Methanol	0.57	0.66
25% Methanol	0.32	0.41
5% Ethanol	0.67	0.67
10% Ethanol	0.52	0.52
25% Ethanol	0.23	0.24
0.5 M NH_4OAc	1.09	1.09
1.0 M NH_4OAc	0.45	0.67
0.25 M KCl	0.79	0.92
0.50 M KCl	0.37	0.63
0.04 M $MgCl_2$[d]	1.15	1.15
0.05 M $MgCl_2$	0.76	0.76

[a] Rate measured over a 15-min period at 20° in binding conditions. [b] pH was maintained at 5.5. [c] Corrected activity is observed fraction activity divided by $(1 - e^{-h})/h$, where h is the mean number of inactivating hits suffered by isoleucyl-tRNA synthetase during the period of incubation. This approximately corrects for inactivation (see text). [d] Total concentrations: 0.01 M $MgCl_2$ is present in normal binding conditions.

Discussion

Under conditions which are quite typical of *in vitro* experiments (moderate temperature and overall ionic strength, $Mg^{2+} \sim 10$ mM), $tRNA^{Ile}$ is more compact, or its structure is more rigid than is optimal for interaction with isoleucyl-tRNA synthetase. On addition of small amounts of organic solvents or dramatic reductions of the Mg^{2+} concentration (Figure 2), the association constant goes up until an optimum is reached. Similarly, stabilizing the structure by adding monovalent or (especially) divalent cations results in a rapid drop in the association constant. These effects cannot be simply enhancement or suppression of an interaction between the negatively charged RNA and a positive site on the protein, for reasons treated in the Results section. It appears in addition that loosening the structure of $tRNA^{Ile}$ or making it more mobile, increases the apparent standard free energy of binding by helping the tRNA to conform to the tRNA site on the aminoacyl-tRNA synthetase. It should be clear that this argument does not imply that ionic forces are not important in binding. It is only true that conformational effects dominate the effects on ionic attractions under these conditions. In other words, this interpretation does not bear directly on the question of whether isoleucyl-tRNA synthetase senses the distribution of charge in the tRNA structure, or some other quality of the tRNA.

There is evidence that other tRNA (*E. coli*)'s may behave in a similar way under changes in solvent, and thus that the behavior of $tRNA^{Ile}$ may be generalized. $(K_m)^{-1}$ in synthetase–tRNA systems is quite often closely related to the true association constant for tRNA, and Loftfield (Loftfield and Eigner, 1967) has shown that $(K_m)^{-1}$ of $tRNA^{Val}$ (*E. coli*) is progressively increased by low concentrations of ethanol; it is also depressed by NaCl addition. This suggests that the $tRNA^{Val}$ (*E. coli*) system used in that work behaves similarly to $tRNA^{Ile}$, even though velocity of aminoacyl transfer to $tRNA^{Val}$ was, unlike $tRNA^{Ile}$, affected strongly by changes in solvent.

A possible further example can be found in the work of Sarin and Zamecnik (1965), who in aminoacylation mixtures containing unresolved *E. coli* tRNA and crude *E. coli* supernatant enzymes, find that the apparent capacity of tRNA for Phe, Tyr, Leu, and Asp (but not for Val and Lys) is increased in the presence of diethylene glycol, and other similar compounds. Under these conditions (crude supernatant enzyme, unresolved and unpurified RNA), an increased affinity of tRNA for its synthetase might in fact give it an advantage and result in increased overall aminoacylation.

There is, in fact, also a considerable subliterature on the effect of various salts on the aminoacylation reaction (*e.g.*, Svenson, 1967; Smith, 1969) which I will not treat. A careful consideration of the two examples above involving organic solvents illustrates the ambiguities in this literature. Examples of systems can be found which respond in any conceivable manner to added salt, but in all cases, aminoacyl transfer has been measured, and this has an uncertain connection to the

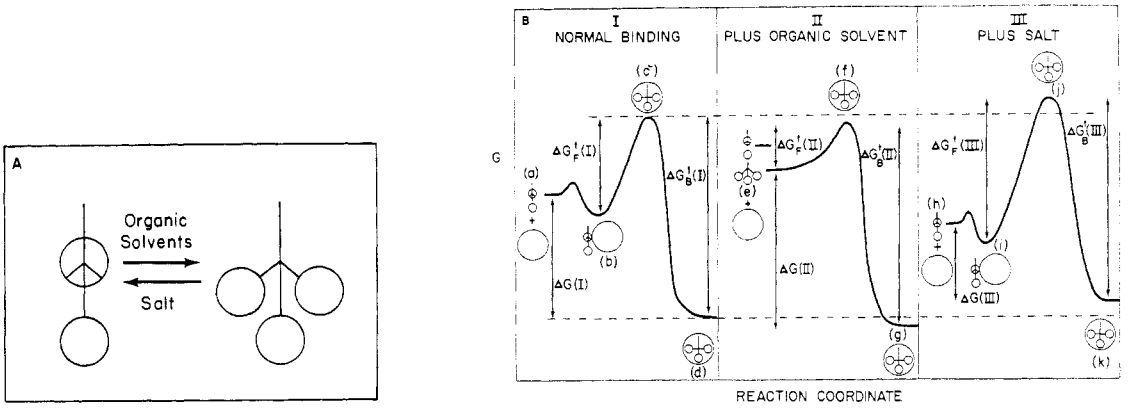

FIGURE 10: A symbolization of these results: a suggested mechanism for binding. (A) The normal and loosened forms of Ile-tRNA and their interrelation. (B) A thermodyanmic interpretation of these results: free energy (G) of the system during binding is represented by the curved lines, large open circles near the curves represent isoleucyl-tRNA synthetase; when touching or covered by a tRNA symbol isoleucyl-tRNA synthetase is in complex with the tRNA.

original interaction (recognition) of the tRNA by the protein concerned. This may be vividly illustrated by comparing Table II and Figure 2 of this work where the association constant of $tRNA^{Ile}$ varies >300-fold in a selected range of conditions in which the velocity of aminoacylation varies less than twofold.

What is the nature of the change in $tRNA^{Ile}$ on addition of 5–10% organic solvent? Clearly a detailed picture will require further study, but from this work, three things are clear: (1) a rather extensive change in shape must occur (Figure 5, but *cf.* 7); (2) this change produces little net change in short-to-medium range order in $tRNA^{Ile}$, that is, little hyperchromicity (Figure 3); and (3) little or no loss of hydrogens shielded from exchange with solvent (Figure 4). This is reminiscent of the first stage of thermal denaturation of yeast tRNA (Henley *et al.*, 1966), in which a large change in shape, a drop in molecular asymmetry, occurs between 20 and 40°, ($\mu \sim 0.2$, no Mg^{2+}) accompanied by a drop in $s_{20,w}$ comparable to that seen here. Again, similarly, in the range where most of this change occurs, only a few per cent of the total hyperchromicity has been expressed.[6] It has also been found, using a fluorescent dye coupled to the 3′ end of unresolved *E. coli* tRNA (Millar and Steiner, 1966), that the total fluorescence and its polarization behave (0.02 M Mg^{2+}) as if molecular transitions having a marked effect on the environment of the 3′ end and the rigidity of the molecule occur below the Tm determined by absorbance. These striking transconformational reactions, then, may be related to the ability of the tRNA to function; their occurrence under other conditions may be no accident.

To provide a framework for discussion of the specificity of the isoleucyl-tRNA synthetase ($tRNA^{Ile}$) interaction, and in order to show that these results may be embodied in a self consistent, plausible model, I have cast the interpretation of this study in graphic and thermodynamic terms (Figure 10).

The most comprehensive current models of the tertiary structure of tRNA (for a review, see (Cramer, 1971) consist, crudely described, of a long stalk composed of the anticodon stem and loop and the CCA stem; more or less in the middle of the molecule is a bulge, sometimes supposed to composed of six RNA strands in close proximity (Cramer, 1971), made by wrapping the dihydro-U and GTψC loop and stem around the stalk which defines the long axis. This folded structure is symbolized in Figure 10A by the diagram on the left, which is meant to evoke the compactly folded nature of this structure without implying a detailed structure or a choice between current models; the arrows symbolize the effect of changes in solvent and it is important to realize that the reaction of Figure 10A is a reaction of free tRNA, and not necessarily one characteristic of the binding reaction. Addition of organic solvents gives the loosened form on the right. While it helps to make the discussion concrete, the right-hand diagram should not be interpreted literally as a picture of a planar cloverleaf. It is instead a symbol for a loosened and more mobile form of tRNA.

Now, these symbols are used in Figure 10B, which is both a hypothetical graph of free energy along the reaction coordinate for binding, and, at intervals along the graphs, a symbolic representation of the state of the tRNA at that point. Panel I, which represents the binding reaction under usual conditions, shows the compact free solution state of $tRNA^{Ile}$ on the left (a). Under these conditions, the tRNA is strongly bound by isoleucyl-tRNA synthetase (represented, when free of tRNA, as a large open circle). Thus the tightly bound state of $tRNA^{Ile}$, state (d), lies below the state of free tRNA, and is therefore bound by $\Delta G(I)$, the usual standard free energy of binding. To reach this strongly bound state, free tRNA must pass through a weakly bound state (b). This state is presumed to exist because the form of tRNA presented to isoleucyl-tRNA synthetase (a) is not the same as that finally bound in (d). This assertion in turn rests on the evidence in this work for an optimal conformation for binding. This state is shown as weakly bound (only slightly negative ΔG with respect to free reactants) because tRNA cannot interact optimally with its site at this stage.

In any case, the rate of the forward reaction is controlled by the activation free energy $\Delta G_F^{\ddagger}(I)$ required for the (b) → (c) transition, which involves transconformational change in the tRNA. The rate of the association is changed by solvents with conformational effects (*cf.* Figure 2, Figure 9, and Table II), and so the diagram embodies the data by making (b) → (c) the rate-limiting step in the forward direction. We will discuss the conformation of the tRNA in the transition state (c) below. Because the binding equilibrium lies strongly to the side of the complex, the free energy of activation required for the dissociation of tRNA, $\Delta G_B^{\ddagger}(I)$, is much larger than $\Delta G_F^{\ddagger}(I)$, and the back rate is correspondingly slow. In the tightly bound

[6] $tRNA^{Ile}$ is very stable to thermal denaturation under usual binding conditions (T_m = 82°[2]). Since Ile-tRNA synthetase becomes unstable at temperatures far less than this, it was not easily possible to attempt to observe thermal effects (but *cf.* Yarus and Berg, 1970).

state (d) in Figure 9b, in which all areas of the tRNA are now in effective contact with isoleucyl-tRNA synthetase, the complex is presumed to be capable of aminoacyl transfer to the tRNA.

Panel II of Figure 9B illustrates the reaction in the presence of low concentrations of organic solvent. First, addition of these solvents changes the conformation of the tRNA (Figures 3 and 5). A physically plausible guess at the mechanism of this effect is shown in the figure; these solvents are depicted destabilizing the more compact forms of tRNA (moving them up on the diagram) more than they do more open forms; thus the tRNA when free in solution now prefers to be in a state inaccessible to it before; that is, a loosened state (e), is now the low lying one for free tRNA. It is now easier ($\Delta G_F^{\ddagger}(II) < \Delta G_F^{\ddagger}(I)$) to reach the transition state (f) because the reaction begins with a higher lying state of the tRNA; for the same reason the overall free energy of binding, $\Delta G(II)$, is larger, as indicated by the data of Figure 2B. In fact, a second type of rate increase, and increase in $\Delta G(II)$, has been drawn into panel II, in that the transition state (f), and final state (g) have been stabilized (lowered). This has been done because there is evidence from the study of small competitors with tRNA[2] that isoleucyl-tRNA synthetase perceives the phosphate group to some extent: therefore (g) would probably be somewhat stabilized when organic solvents are added and the dielectric constant is lowered. However, it is extremely important to note that the data shows that the rate of release of Ile-tRNA is unaffected by organic solvents (as implied by Figure 9 and Table II). This implies that the spacing between states (f) and (g) must be the same as between (c) and (d). That is, $\Delta G_B^{\ddagger}(I) \simeq \Delta G_F^{\ddagger}(II)$. Thus, in order to be consistent with the data, solvent variation must either not affect the transition and final states, which seems improbable, or must affect them equally. The tRNA in (e) and (f) have been drawn differently to reemphasize that we do not know the relation between the state of the free and bound tRNA.

Finally, panel III interprets the data for binding in conditions in which salt has been added. These stabilize the compact, free form of tRNA (h) so that the reaction begins lower on the diagram than the reactants in (a). The (a) → (b) transition is analogous to the (h) → (i) transition; so it will be true that $\Delta G_F^{\ddagger}(III) > \Delta G_F^{\ddagger}(I)$ and the forward reaction in salts will be slower (as suggested by Figure 2A and Table II), and $\Delta G(III) < \Delta G(I)$, so tRNA is more weakly bound overall (Figure 2A). Again (from Table II), while it appears plausible that (j) and (k) must be affected by solvents, their spacing, $\Delta G_B^{\ddagger}$, must be the same: $\Delta G_B^{\ddagger}(I) \simeq \Delta G_B^{\ddagger}(II) \simeq \Delta G_B^{\ddagger}(III)$. Since it appears in a wide variety of conditions that the rate of dissociation is unaffected by changes which should affect both coulomb forces and tRNA conformation, these two qualities of the transition state probably resemble quite closely the same qualities of the final tightly bound state. Thus I surmise that neither transconformational changes in tRNA nor net change in ion–ion forces are involved in traversing (k) ↔ (j), (g) ↔ (f), or (d) ↔ (c). For that reason the same symbol has been used for the transition and final states, and these states have been drawn identically in panels I, II, and III. This conclusion is independent of the particular interpretation put forward for the effect of solvents on the forward reaction.

Let us turn now to the specificity of the interaction of $tRNA^{Ile}$ and isoleucyl-tRNA synthetase. We take specificity to mean the *ratio* of the strength of binding, or $\Delta G(I)$, measured for a cognate and, on the other hand, for a noncognate tRNA. If panel I of Figure 9B were redrawn, then, for the interaction of Ile-tRNA synthetase with a noncognate tRNA, we would as a first approximation, start at the same level as state (a), because tRNAs are quite similar in structure and sequence; however, we would not achieve an effective interaction: that is $\Delta G(I)$ would be small, though not zero;[2] because the most tightly bound state, whatever its structure, will lie considerably above (d). However, now consider the situation represented in II. Because, again, tRNAs are generally similar in structure we expect that some or perhaps all noncognate tRNAs can also be forced into states which lie above their usual compact free solution states, as $tRNA^{Ile}$ was in panel II. As a result, $\Delta G(II)$ for the noncognate pair will increase. If it increases enough, they could achieve an effective interaction: that is, the specificity of the interaction, as defined above, would decline and the noncognate tRNA would begin to bind to the enzyme; in a sufficiently fortunate case, even misaminoacylation might be achieved. From this point of view, then, the specificity of the interaction arises in part from the nature of the higher order structure of tRNA; it is so contrived by evolution to deny the tRNA access to states from which a successful interaction between noncognates can be launched. One could concisely express this view by saying that there is a conformational barrier to reaction between noncognates; or that part of the specificity is achieved through the stability of the compact structure under normal conditions. It should be realized that the "barrier" referred to in this statement is an unusual one: its bottom (Figure 10), rather than its top, is the obstacle to a mistaken interaction.

There is already data in the literature which may be interpreted as confirmation of this point of view. The cytoplasmic phenylalanyl-tRNA synthetase of *Neurospora* can mistakenly recognize and aminoacylate $tRNA^{Val}$ (*E. coli*) (for a review, see Jacobson (1971)), and the overall rate and extent of this reaction are very sensitive to the conditions under which the reaction is conducted (Jacobson, 1971). For example, Tris-HCl-buffered reactions are generally more limited than those conducted in cacodylate, perhaps in part because the $(K_m)^{-1}$ of enzyme for tRNA is 100× smaller in Tris (Jacobson, 1971). Under these conditions, ethanol and dimethyl sulfoxide markedly stimulate the reaction (Ritter *et al.*, 1970), and NH_4Cl and NaCl addition produce dramatic suppression. While the reasons for dependence of the extent of reaction on conditions are still obscure, these data suggest that a loosening reaction may comprise part of the barrier to reaction between this transcognate and transpecific pair. To convincingly evaluate the role of solvent in selectivity among tRNAs, direct measurements are needed on the effect of solvent loosening on the level of discrimination among tRNAs in an intraspecific system. These experiments are now underway, and I have, in fact, been able to show that misrecognition and misaminoacylation can occur under conditions characterized here as loosening tRNA structures, and thus been able to gain expermental control of the specificity of this nucleic acid–protein interaction (Yarus, 1972).

Finally, I point to the implications of these results for the crystallography of tRNA and its relation to tRNA function. Many solvents used to reduce tRNA solubility during crystallization contain organic solvents (*e.g.*, Clark *et al.*, 1968, and Kim and Rich, 1969). While it is not yet known how similar the response of different tRNAs may be, these solvents clearly may change the structure which exists in a completely aqueous environment, and the X-ray structure may not be identical to that in solution. On the other hand, even the hypothetical recollapsed form of the tRNA in 25% dioxane, is recognizable to isoleucyl-tRNA synthetase (Figure 2B), and so is physio-

logical in one sense. However, because it appears that changes occur during binding, neither of these types of structure necessarily simulates closely that structure which exists on the synthetase: thus cocrystals of synthetase and site-bound tRNA may ultimately be required to understand their interaction. Unfortunately, the large effect of ionic strength on the association constant (Figure 2B) will make cocrystals elusive by standard methods of protein crystallization, which involve salting out.

Acknowledgments

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References

Baldwin, A. N., and Berg, P. (1966), *J. Biol. Chem. 241*, 831.

Clark, B. F. C., Doctor, B. P., Holmes, K. C., Klug, A., Marcker, K. A., Morris, S. J., Paradies, H H. (1968), *Nature (London) 219*, 1222.

Cramer, F. (1971), *Progr. Nucl. Acid Res. Mol. Biol. 11*, 391.

Edelstein, S. J., and Schachman, H. K. (1967), *J. Biol. Chem. 242*, 306.

Eldred, E. W., and Schimmel, P. R. (1972), *Biochemistry* (in press).

Englander, S. W. (1968), *Methods Enzymol. 12B*, 379.

Englander, S. W., and Englander, J. J. (1965), *Proc. Nat. Acad. Sci. U. S. 53*, 370.

Gantt, R. R., Englander, S. W., and Simpson, W. V. (1969), *Biochemistry 8*, 475.

Gillam, I., Millward, S., Blew, D., Von Tigerstrom, M., Wimmer, E., and Tener, G. M. (1967), *Biochemistry 6*, 3042.

Hanson, C. V. (1971), *J. Mol. Biol. 58*, 847.

Henley, D. D., Lindahl, T., and Fresco, J. R. (1966), *Proc. Nat. Acad. Sci. U. S. 55*, 191.

Herskovits, T. T. (1962), *Arch. Biochem. Biophys. 97*, 474.

Inagami, T., and Sturtevant, J. M. (1960), *Biochim. Biophys. Acta 38*, 64.

Jacobson, K. B. (1971), *Progr. Nucl. Acid Res. Mol. Biol. 11*, 461.

Kim, S. H., and Rich, A. (1969), *Science 166*, 1621.

Laidler, K. J., and Ethier, M. C. (1953), *Arch. Biochem. Biophys. 44*, 338.

Latt, S. A., and Sober, H. A. (1967), *Biochemistry 6*, 3292.

Levine, L., Gordon, J. A., and Jencks, W. P. (1963), *Biochemistry 2*, 168.

Loftfield, R. B., and Eigner, E. A. (1967), *J. Biol. Chem. 242*, 5355.

Millar, D. B., and Steiner, R. F. (1966), *Biochemistry 5*, 2289.

Nishimura, S., and Novelli, G. D. (1963), *Biochem. Biophys. Res. Commun. 11*, 161.

Ritter, P. O., Kull, F. J., and Jacobson, K. B. (1970), *J. Biol. Chem. 245*, 2114.

Sarin, P. S., and Zamecnik, P. C. (1965), *Biochem. Biophys. Res. Commun. 19*, 198.

Schrier, E. E., Ingwall, R. T., and Scheraga, H. A. (1965), *J. Phys. Chem. 69*, 298.

Smith, D. W. E. (1969), *J. Biol. Chem. 244*, 896.

Svenson, I. (1967), *Biochim. Biophys. Acta 132*, 239.

Tanford, C. (1968), *Advan. Protein Chem. 23*, 121.

Timmermans, J. (1960), Physico-Chemical Constants of Binary Systems, Vol. 4, New York, N. Y., Interscience.

Yarus, M. (1972) *Biochemistry 11* (in press).

Yarus, M., and Barrell, B. G. (1971), *Biochem. Biophys. Res. Commun. 43*, 729.

Yarus, M., and Berg, P. (1967), *J. Mol. Biol. 28*, 479.

Yarus, M., and Berg, P. (1969), *J. Mol. Biol. 42*, 171.

Yarus, M., and Berg, P. (1970), *Anal. Biochem. 35*, 450.

Yarus, M., and Rashbaum, S. (1972), *Biochemistry 11*, 2043.

Yphantis, D. A. (1964), *Biochemistry 3*, 297.

Zubay, G. (1962), *J. Mol. Biol. 4*, 347.