NMR Analysis of Bovine tRNA<sub>Trp</sub>

CONFORMATION DEPENDENCE OF Mg<sup>2+</sup> BINDING<sup>a</sup>

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NMR was used to study the solution structure of bovine tRNA<sub>Trp</sub> hyperexpressed in Escherichia coli. With the use of <sup>15</sup>N labeling and site-directed mutagenesis to assign overlapping resonances through the base pair replacement of U<sup>71</sup>A<sup>2</sup> by G<sup>C71</sup>, U<sup>77</sup>A<sup>43</sup> by G<sup>C77</sup>C<sup>43</sup>, and G<sup>C23</sup>C<sup>52</sup> by U<sup>12</sup>A<sup>23</sup>, the resonances of all 26 observable imino protons in the helical regions and in the tertiary interactions were assigned unambiguously by means of two-dimensional nuclear Overhauser effect spectroscopy and heteronuclear single quantum coherence methods. When the discriminator base A<sup>73</sup> and the G<sup>C23</sup>C<sup>52</sup> base pair on the D stem, two identity elements on bovine tRNA<sub>Trp</sub> that are important for effective recognition by tryptophanyl-tRNA synthetase, were mutated to the ineffective forms of G<sup>73</sup> and U<sup>12</sup>A<sup>23</sup>, respectively, NMR analysis revealed an important conformational change in the U<sup>12</sup>A<sup>23</sup> mutant but not in the G<sup>73</sup> mutant molecule. Thus A<sup>73</sup> appears to be directly recognized by tryptophanyl-tRNA synthetase, and G<sup>C23</sup>C<sup>52</sup> represents an important structural determinant. Mg<sup>2+</sup> effects on the assigned resonances of imino protons allowed the identification of strong, medium, and weak Mg<sup>2+</sup> binding sites in tRNA<sub>Trp</sub>. Strong Mg<sup>2+</sup> binding modes were associated with the residues G<sup>7</sup>, s<sup>4</sup>U<sup>19</sup> (where s<sup>4</sup>U is 4-thiouridine), G<sup>12</sup>, and U<sup>52</sup>. The observations that G<sup>42</sup> was associated with strong Mg<sup>2+</sup> binding in only the U<sup>12</sup>A<sup>23</sup> mutant tRNA<sub>Trp</sub> but not the wild type or G<sup>73</sup> mutant tRNA<sub>Trp</sub> and that the G<sup>7</sup>, s<sup>4</sup>U<sup>19</sup>, G<sup>23</sup>, and G<sup>52</sup> imino protons are associated with a two-site Mg<sup>2+</sup> binding mode in wild type and G<sup>73</sup> mutant but only a one-site mode in the U<sup>12</sup>A<sup>23</sup> mutant established the occurrence of conformational change in the U<sup>12</sup>A<sup>23</sup> mutant tRNA<sub>Trp</sub>. These observations also established the dependence of Mg<sup>2+</sup> binding on tRNA conformation and the usefulness of Mg<sup>2+</sup> binding sites as conformational probes. The thermal titration of tRNA<sub>Trp</sub> in the presence and absence of 10 mM Mg<sup>2+</sup> indicated that overall tRNA<sub>Trp</sub> structure stability was increased by more than 15 °C by the presence of Mg<sup>2+</sup>.

Although the three-dimensional structures of a number of free and enzyme-bound tRNA molecules have been elucidated with x-ray crystallography (1–6), the solution structures of most tRNAs remain undetermined despite their pivotal importance in protein synthesis where they must be recognized with high fidelity by cognate aminoacyl-tRNA synthetases (7). This high fidelity could be achieved through the specific recognition by the synthetase of base sequences unique to the substrate tRNA, the singular solution structure of the tRNA, or both (8). It is therefore necessary to characterize for every tRNA-synthetase system the roles of identity elements on the tRNA that are essential for recognition by the synthetase.

NMR spectroscopy has been systematically applied to conformational analysis of tRNAs based on the finding that resonances from hydrogen-bonded GN1 and UN3 imino protons in RNA base pairs can be detected between 10 and 15 ppm in the 1H NMR spectra, well resolved from other RNA protons that cluster between 3 and 9 ppm (9–13). However, the assignments of the imino proton resonances remain problematic because of overlapping signals. Multidimensional NMR using stable isotopes such as <sup>15</sup>N and <sup>13</sup>C facilitates spectral assignments and the study of interactions between tRNAs and cognate synthetases (7, 14). In a recent study, we found that designed mutagenesis of tRNA sequence provides a particularly powerful technique for the resolution of overlapping NMR signals in tRNA (15). By mutagenizing a base pair with a resonance that overlaps with that of another base pair, the latter resonance may be analyzed unambiguously. Use of this approach has made possible the assignment of almost all of the imino protons in the helical regions and the tertiary base pairs in Bacillus subtilis tRNA<sub>Trp</sub>.

Magnesium ions are essential to tRNA function, and their binding to tRNA has long been investigated (16). In tRNA molecules, weak nonspecific Mg<sup>2+</sup> binding sites are abundant, primarily based on electrostatic interactions of the ion with backbone phosphates, and relatively weak in binding affinities. Strong binding sites are coordinated, either directly or via water, to phosphates and other ligands (17–19). Since the strong Mg<sup>2+</sup> binding sites are non-randomly distributed and also few in number, their locations are expected to depend on RNA structure. The distributions of such strong Mg<sup>2+</sup> sites therefore may furnish potentially useful structural information.

Given the extensive imino proton assignments made possible by a combination of tRNA sequence mutagenesis and 2D NMR (15), the possible conformational roles of the A<sup>73</sup> and G<sup>C23</sup> identity elements on bovine tRNA<sub>Trp</sub> were examined in the present study based on their mutation to the ineffective forms of G<sup>73</sup> and U<sup>12</sup>A<sup>23</sup>, respectively, and monitoring NMR chemical shift changes of different imino protons in the wild type and mutant molecules. Conformational changes were also detected through changes in the behavior of strong Mg<sup>2+</sup> binding sites.

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The abbreviations used are: 2D, two-dimensional; 1D, one-dimensional; TrpRS, tryptophanyl-tRNA synthetase; HSQC, heteronuclear single quantum coherence; NOE, nuclear Overhauser effect; NOESY, NOE spectroscopy; s<sup>4</sup>U, 4-thiouridine.

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MATERIALS AND METHODS

tRNA<sub>T</sub> Preparation and Assay—Bovine tRNA<sub>T</sub> (Fig. 1) was produced from hyperexpressing strains of <i>E. coli</i> JM109 transformed by recombinant pGEM-9zf-derived plasmid containing synthetic bovine tRNA<sub>T</sub> gene and grown in M9-glycerol medium supplemented with 100 μg of ampicillin/ml (15). The tRNA<sub>T</sub> was purified as described by Xue et al. (20). 15N-Labeled tRNA<sub>T</sub> was obtained similarly except that NH<sub>4</sub>Cl was replaced by 15NH<sub>4</sub>Cl (Isotec Inc.) in the growth medium. In addition to wild type bovine tRNA<sub>T</sub>, the single base or base pair mutants of G<sup>73</sup>, G<sup>C71</sup>, G<sup>27</sup>C<sup>43</sup>, and U<sup>12</sup>A<sup>23</sup>, in which the bases at positions 73, 2/71, 27/43, and 12/23 were changed to the designated forms, were also hyperexpressed, labeled, and purified. Tryptophanylation of the wild type and mutant tRNA<sub>T</sub> was carried out with human TrpRS purified as described by Guo et al. (21) using the assay method of Xue et al. (22).

NMR Spectroscopy—All NMR spectra were recorded on a Varian INOVA 500 spectrometer at a probe temperature of 30 °C. Jump-and-return sequence was applied in all 1D and 2D NOESY spectra for suppressing solvent signal as described by Yan et al. (15). Phase-sensitive 2D NOESY spectra were recorded at a 120-ms mixing time with the hypercomplex method for quadrature detection in F1 dimension. A total of 256 t<sub>1</sub> experiments with 4096 real points were collected over a spectral width of 12,000 Hz in each dimension. Sensitivity-enhanced gradient 2D 15N-1H HSQC spectra were recorded with a spectral width of 12,000 and 6000 Hz in the proton and nitrogen dimensions, respectively. One hundred and twenty-eight t<sub>1</sub> increments were collected, each with 2048 real points. For NMR studies, 13–18 mg of tRNA<sub>T</sub> as determined by UV absorbance at 254 nm was dissolved in 0.5 ml of Buffer A containing 10 mM MgCl<sub>2</sub>, 100 mM sodium chloride, and 10 mM sodium phosphate, pH 6.5. D<sub>2</sub>O was added to 8% as a lock signal.

<sup>15</sup>N Binding Curve—Magnesium ion was removed from the tRNA samples by dissolving the tRNA in 2 ml of Buffer A containing 100 mM EDTA in place of 10 mM MgCl<sub>2</sub> and heating to 50 °C for 5 min. Afterward the solution was concentrated to 20% of the volume using Centracron-10 (Amicon Inc.), washed two to three times with the same EDTA-containing Buffer A, and washed three to four times more with the same buffer without EDTA. The final volumes of tRNA samples were adjusted to 0.45 ml. Titration with Mg<sup>2+</sup> was achieved by adding successively small aliquots (5 μl) of a series of MgCl<sub>2</sub> solutions of appropriated concentrations directly to the NMR tube. To construct binding curves of Mg<sup>2+</sup> to tRNA, the chemical shifts of individual imino protons were plotted as a function of Mg<sup>2+</sup> concentration. Most data could be fitted to a one-binding-site model by means of software program Xcrvfit (Protein Engineering Network of Centres of Excellence (PENCE)/Medical Research Council of Canada (MRC) Group Joint Software Centre, Edmonton, Alberta, Canada). For binding curves with a clear departure from hyperbolic behavior in the form of a maximum, the data were fitted to a two-binding-site model by means of the same software (23).

RESULTS

Imino Proton Resonances

The usual assignment strategy for the imino proton resonances of nucleic acids was applied to the hydrogen-bonded segments of tRNA. The imino protons in these segments are close enough (≤5 Å) to give NOEs so that assignment of these protons can be achieved via a chain of connectivities provided a suitable starting point or a unique sequence is available (24). Imino protons involved in hydrogen bonding in base pairs and tertiary interactions are protected from exchange with solvent and therefore visible in the downfield region of the 1H NMR spectrum (25). Typically ~28 such imino protons resonances are expected to appear for a canonical tRNA. For wild type bovine tRNA<sub>T</sub>, 28 imino protons were distinguished in the region of the 1H NMR spectrum between 9 and 15 ppm (Table I). Two-dimensional 15N-1H HSQC (Fig. 2) and NOESY (Fig. 3) were both used for imino proton assignment. The 15N labeling allowed ready differentiation between UA (15N shifts 156.8 ppm for UN<sub>3</sub>) and GC (15N shifts 142.9 ppm for GC<sub>3</sub>) base pairs (24).

Acceptor Stem—There is no special base in the acceptor stem that can be used as a starting marker for resonance assignment. To identify base pairs in this stem, a 15N-labeled tRNA<sub>T</sub> mutant was prepared in which U<sup>17</sup>A<sup>41</sup> was replaced by G<sup>C71</sup>. The 15N-1H HSQC spectrum of this G<sup>C71</sup> mutant indicated clearly that an imino proton resonance originally located...
at 14.24 ppm in the UA base pair region disappeared, accompanied by the emergence of a new resonance at 12.16 ppm in the GC base pair region. Based on this observation, the imino proton of G^{34} was unambiguously assigned. This imino proton gave NOEs to two different GC base pairs at 11.77 and 13.16 ppm, respectively. Since the base pair at 11.77 ppm was very weak in intensity, it was assigned to the imino proton of G^{47}C^{52}, which, being located at the open end of the acceptor stem, might be expected to undergo significant unstacking. The second base pair, which thereupon could be regarded as the imino proton of G^{47}C^{52}, gave rise to a further NOE to the G^{50}C^{5} base pair. Because GU base pairs contain two hydrogen-bonded imino protons that are strongly dipolar-coupled on account of their close proximity (<3 Å), they usually yield the strongest cross-peaks in the NOESY spectrum. On this basis the two GU base pairs in wild type bovine tRNA_{Trp} were therefore linked to the two strongest resonances in the upfield imino proton region. One of the base pairs gave NOEs to the identified G^{50}C^{5}, and the next GC base pair in the stem (namely G^{57}C^{62}) was assigned to G^{63}U^{5}. At the end of the acceptor stem, the imino proton of the G^{60}C^{66} base pair was also connected in turn by NOE to the imino proton of G^{67}C^{6}. Thereby the base pairs in the acceptor stem were completely assigned.

Ribothymidine Stem—Like many other canonical tRNAs, bovine tRNA_{Trp} contains a sole ribothymidine residue at position 54. The thymidine methyl group resonates remarkably in the upfield region of 1H NMR spectrum at 0.99 ppm and is thus easily recognized. Since the proton of the methyl group is close to φ^{-1/2} and formed the reversed Hoogsteen pair T^{34}A^{36} crossing the ribothymidine loop, it gave a set of NOE cross-peaks to their imino protons. This characteristic NOE crossing pattern in two-dimensional NOESY helped to assign the imino protons of T^{34} and ψ^{55} (15). The identified T^{34} imino proton showed a further NOE connectivity to a GC base pair, assignable to the spatially adjacent G^{50}C^{51}. The imino proton of G^{51} in turn gave a weak NOE to the AU base pair U^{62}A^{62}, which was confirmed in the 15N-H HSQC spectrum. The next NOE cross-peak between the imino protons of U^{62} and G^{51} could be traced out via T^{54}C^{56} and onward through C^{53}U^{52} NOE cross-peaks. Because there was no more distinct sequential NOE cross-peak available, the last two base pairs could be assigned only from the other end of the ribothymidine stem. Of the two GU base pairs in the tRNA, G^{68}U^{65} in the acceptor stem was already assigned. Thus the remaining GU base pair could be identified unambiguously as G^{49}U^{65}. Based on the NOE cross-peak between G^{49}U^{65} and the adjacent G^{64}C^{50} base pair, the imino proton of G^{64} also could be assigned. The imino protons of G^{49} and G^{51} were too similar in chemical shift to display a distinguishable NOE peak from diagonal peaks.

Anticodon Stem—In addition to the three identified UA base pairs in the 15N-H HSQC spectrum, only one characteristic UA base pair remained to be assigned at 13.08 ppm, which could be attributed unambiguously to U^{29}A^{41} in the anticodon stem. The imino proton of this UA base pair gave NOEs to two GC base pairs at 12.38 and 11.65 ppm. No further sequential NOE connectivities related to these base pairs was observed. This might be due to the fact that since the UA and ψA base pairs are located at the two ends of the anticodon stem, dynamic fluctuations could cause them to become unstacked. To identify the GC base pairs in the anticodon stem, 15N-labeled mutant tRNA_{Trp} with a base pair change from U^{27}A^{43} to G^{27}C^{43} was cloned, expressed, and purified. In the 15N-H HSQC spectrum, a new GC imino proton resonance appearing at 13.16 ppm was assigned to this G^{27}C^{43}. A GC base pair originally located at 12.38 ppm in the wild type moved to 12.77 ppm in the G^{27}C^{43} mutant, but no change in chemical shift was observed on another GC base pair located at 11.65 ppm. The 12.38 ppm GC base pair consequently could be assigned to the G^{23}C^{25} base pair adjacent to U^{27}A^{43}, leaving the 11.65 ppm resonance assignable to G^{10}C^{30}.

Dihydouridine Stem—Another commonly encountered reversed Hoogsteen pair in tRNA, s^{4}U^{8}A^{14}, provided an independent starting point for dihydrouridine stem assignments. Since the imino proton of this tertiary base resonated downfield to all other imino protons in both the 1H and 15N dimensions on account of deshielding in thiouridine, it was readily identified at 15N shift 182.4 ppm. Based on the NOE between imino protons of s^{4}U^{8}A^{14} and its immediate neighbor of the G^{22}C^{13} base pair (15), the G^{22} imino proton could be assigned to 13.06 ppm. However, no further NOE linked with G^{22}C^{13} could be detected in the NOESY spectrum. This suggests that the G^{22}G^{22} NOE cross-peak was overlapped by diagonal signals because of the close similarity in chemical shifts between the imino protons of G^{22}C^{13} and G^{23}C^{25} base pairs. Three unidentified GC base pairs remained to be assigned in the 15N-H HSQC spectrum. Two potential G^{12}C^{25} base pairs resonated respectively at 12.96 and 13.22 ppm, yet another GC base pair at 12.72 ppm was connected to the 13.22 ppm base pair by NOE. To distinguish between these three GC base pairs, the 15N-labeled U^{12}A^{23} tRNA_{Trp} mutant containing U^{12}A^{23} in place of G^{12}C^{23} was prepared and analyzed. In the 15N-H HSQC spectrum of this U^{12}A^{23} mutant, the wild type GC resonances
at 12.96, 13.06, and 13.22 ppm, which would include that of $G^{22}C^{13}$, were missing from their original positions. Information from the NOESY spectrum suggests that the unaltered 12.72 ppm GC base pair could be assigned as $G^{10}C^{25}$, and the 13.22 ppm GC base pair to which $G^{10}C^{25}$ was linked by NOE could be assigned as $G^{12}C^{11}$. Since $G^{22}C^{13}$ at 13.06 ppm was already assigned based on its NOE to $s^4U^8A^{14}$, the remaining GC base pair at 12.96 ppm was therefore assigned to $G^{12}C^{23}$.

Besides the two assigned reversed Hoogsteen pairs of $s^4U^8A^{14}$ and $T^{54}A^{58}$, wild type tRNA$^{Trp}$ also contained the conserved tertiary $\psi^{92}G^{18}$ base pair between the T loop and D loop. The imino proton of $G^{18}$ resonated at 9.32 ppm, upfield from all other imino proton resonances, and gave an NOE to the $\psi^{92}N^3$ proton at 11.34 ppm, whereas the $\psi^{92}N^1$ proton was assigned on the basis of the mutual NOE at 10.34 ppm between $N1$ and $N3$ protons (26). The two possible UA resonances at 11.64 and 10.67 ppm in the $^{15}N$-$^1H$ HSQC spectrum, devoid of any detectable NOE connectivities, yet require further identification.

Among the four $^{15}$N-labeled tRNA$^{Trp}$ mutants $G^{73}$, $G^{2C71}$, $G^{27}C^{43}$, and $U^{12}A^{23}$, $G^{73}$ gave 1D spectra most similar to the wild type. Most peaks in $G^{2C71}$ also could be overlapped by those in wild type except for the evident loss of a UA resonance at 14.24 ppm and the emergence of a GC resonance at 12.16 ppm as expected from the UA to GC mutation. Both $G^{27}C^{43}$ and $U^{12}A^{23}$ underwent minor spectral changes, this being especially the case with the $U^{12}A^{23}$ mutant (Fig. 4). Since the major spectral features of all these mutants largely resemble those of the wild type, all four mutant molecules must share with the wild type a closely similar three-dimensional conformation. The individual imino protons in the different mutant structures could be assigned simply by comparing the mutant and

FIG. 3. **The low field region of the 2D $^1H$ NOESY spectrum of bovine tRNA$^{Trp}$ wild type at 30 °C.** All identified NOE cross-peaks at acceptor stem, D stem, anticodon stem, and T stem are connected by blue, black, green, and red straight lines, respectively.

FIG. 4. **The 1D imino proton NMR spectra of bovine wild type (a), $G^{73}$ (b), $G^{2C71}$ (c), $G^{27}C^{43}$ (d), and $U^{12}A^{23}$ (e) at 30 °C.**
Magnesium Ion Binding

Mg$^{2+}$ binding curves for wild type tRNA$^{\text{Trp}}$ and the G$^{73}$ and U$^{12A}$A$^{55}$ mutants were obtained by addition of Mg$^{2+}$ to the tRNA molecules and monitoring the changes in the assigned imino proton chemical shifts. In the wild type and the G$^{73}$ mutant, the imino protons of U$^{65}$ and $\psi^{55}$ in the T stem displayed the largest upfield shift changes followed by those of G$^{67}$ of the acceptor stem, G$^{32}$ of the T stem, and U$^{29}$ of the anticodon stem with larger downfield shifts (Fig. 5). These differences in magnitude of Mg$^{2+}$-induced chemical shift changes could be the result of changes in the local environment of the proton or structural changes in the tRNA molecules (27). The G$^{11A}$A$^{33}$ mutant displayed a similar pattern of chemical shift changes with additional large downfield shifts for the imino protons of s$^{5}$U$^{5}$ and G$^{34}$ in the D stem.

The majority of Mg$^{2+}$ binding curves determined from the various assigned imino protons was hyperbolic and could be fitted to a one-binding-site model. Some of the curves, however, displayed a maximum and required fitting to a two-binding-site model (Table II). Because of the lack of a satisfactory procedure for calculating free Mg$^{2+}$ concentration in the face of multiple metal ion binding events (28, 29), no attempt was made to estimate the exact Mg$^{2+}$ binding dissociation constants. The fitted curves for imino protons associated with tight and medium Mg$^{2+}$ binding sites are shown in Fig. 6.

**Wild Type and G$^{73}$ Mutant**—In both the wild type and G$^{73}$ mutant, many of the imino protons conformed to hyperbolic titration curves of tight binding with a half-saturation Mg$^{2+}$ concentration, or $K_{1/2}$, of 1–10 mM, medium binding with $K_{1/2}$ of 10–20 mM, weak binding with $K_{1/2}$ of 20–500 mM, or marginal binding with $K_{1/2}$ of >500 mM (Table II). The chemical shift changes of G$^{68}$ and G$^{69}$ imino protons were too small to give accurate binding curves, whereas U$^{52}$ in the T stem and G$^{12}$ in the D stem displayed exceptionally strong binding (Fig. 6). On the other hand, the Mg$^{2+}$ binding curves of the G$^{7}$, s$^{5}$U$^{5}$, G$^{24}$, and G$^{22}$ imino protons were obviously non-hyperbolic and thus required fitting by a two-binding-site model. Among them, G$^{7}$, s$^{5}$U$^{5}$, and G$^{24}$ exhibited strong binding of the first magnesium ion with low $K_{1/2}$ values in the range of 1–10 mM (Fig. 6).

**U$^{12A}$A$^{55}$ Mutant**—The U$^{12A}$A$^{55}$ mutant displayed a number of differences from the wild type. Its imino protons from U$^{12}$, G$^{49}$, and G$^{51}$ were unexpectedly missing, probably overlapped by other resonances in the $^{15}$N-$^1$H HSQC spectrum. Remarkably, besides its imino protons of U$^{5}$, G$^{65}$, G$^{1}$, and G$^{22}$, which scarcely changed their chemical shifts during the Mg$^{2+}$ titration, the titration curves of all its observed imino protons were hyperbolic and could be fitted by the one-site model. This behavior of the U$^{12A}$A$^{55}$ mutant was a sharp departure from the wild type or G$^{73}$ mutant in which the response of four imino protons to Mg$^{2+}$ binding required a two-binding-site model for description. While the s$^{5}$U$^{5}$ and G$^{24}$ imino protons in U$^{12A}$A$^{55}$ remained associated with tight Mg$^{2+}$ binding, the U$^{55}$ imino proton in the ribothymidine stem and G$^{24}$ imino proton in the anticodon stem were indicative of much stronger Mg$^{2+}$ binding compared with the wild type and G$^{73}$ molecules (Fig. 6). Similarly an increase in Mg$^{2+}$ binding affinity also was evident with $\psi^{55}$N$^{1}$ and $\psi^{55}$N$^{3}$, which were marginal sites of Mg$^{2+}$ binding in both wild type and G$^{73}$ mutant.

Temperature Effects

The $^1$H NMR spectra of wild type bovine tRNA$^{\text{Trp}}$ in the presence or absence of Mg$^{2+}$ at different temperatures are shown in Fig. 7. In the presence of 10 mM Mg$^{2+}$, most of the peaks changed their chemical shifts upfield with increasing temperature and gave indications of melting with peak intensity reduction and signal broadening. Comparing the 2D $^{15}$N-$^1$H HSQC spectra obtained at different temperatures, the signals due to U$^{71}$ and U$^{52}$ were found to weaken at 40 °C and almost vanish at 50 °C. The signal of $\psi^{55}$N$^{1}$, which is not hydrogen-bonded, became broadened at 40 °C and totally disappeared at 60 °C. The peak intensities of G$^{1}$ and G$^{18}$ decreased sharply at 50 °C and disappeared at 60 °C. The peaks of U$^{29}$ and G$^{30}$ in the anticodon stem displayed reduced intensities at 50 °C. Almost all resonances underwent considerable reduction in intensity and peak broadening at 60 °C, especially those of the two GU base pairs G$^{49}$U$^{55}$ and G$^{65}$U$^{51}$. The total melting of tRNA structure at 70 °C was indicated by the disappearance of all imino resonances.

These observations suggest that melting began at 40 °C at the 3′-end of acceptor stem and the U$^{52A}$A$^{62}$ region of T stem and gradually spread to the entire anticodon stem, the acceptor stem, and the T stem. That only NOE connectivities of the D stem remained observable in 2D NOESY at 50 °C points to a higher degree of stacking in the D stem, thus resisting against extensive melting up to 70 °C. The tertiary interaction between $\psi^{55}$ in T loop and G$^{18}$ in D loop was disrupted completely at 60 °C, whereas the other two tertiary base pairs s$^{5}$U$^{5}$A$^{14}$ and T$^{4A}$A$^{56}$ did not vanish until 70 °C. Thus the tertiary base pair G$^{18}$ $\psi^{55}$, being located at the outer corner of the L-shaped structure of the tRNA molecule (1, 2), evidently became more readily accessible to solvent exchange with increasing temperature.

In the absence of Mg$^{2+}$, the imino proton peaks of the terti-
ary interactions $s^1U^8A_{14}$ and $G^{18}_{55}$ were already diminished at 30 °C. At 40 °C, most of the imino proton signals lost substantial intensities. However, the $U^{71}, G^{70}, G^{69}, U^5,$ and $G^{68}$ peaks in the acceptor stem, although reduced, were clearly visible. Although the tertiary interactions and the other stems melted by 50 °C, the acceptor stem remained largely intact. By 60 °C, all the imino proton signals in the tRNATrp disappeared (Fig. 7, left and right).

### TABLE II

The residues of bovine tRNA$^{Trp}$ involved in the Mg$^{2+}$ bindings

The residues whose imino proton require a two-binding-site model for description are shown in bold. The titration curves of the imino protons from $U^5$, $G^{67}$, $U^{29}$, and $G^{55}$ in wild type and $G^{73}$ mutant and $G^{70}$ and $U^{29}$ in $U^{12}A^{23}$ mutant remained largely linear to high Mg$^{2+}$ concentration, pointing to a very low affinity (>500 mM) interaction. The chemical shift changes of $G^{68}$ and $G^{49}$ imino protons in wild type and $G^{73}$ mutant and $U^5$, $G^{68}$, $G^{22}$, and $G^{49}$ in $U^{12}A^{23}$ mutant are too small to be used to estimate the $K_1$. These residues therefore are not shown in the table. The imino protons of $G^{12}$, $G^{49}$, and $G^{51}$ are missing in the $^{15}$N-$^1$H HSQC spectrum of $U^{12}A^{23}$ mutant; the $K_1$ of these imino protons could not be measured.

| tRNA$^{Trp}$ | Strong binding ($K_1$ of 1–10 mM) | Medium binding ($K_1$ of 10–20 mM) | Weak binding ($K_1$ of 20–500 mM) | Marginal binding ($K_1 > 500$ mM) |
|--------------|-----------------------------------|-----------------------------------|-----------------------------------|----------------------------------|
| WT           | $G^7, s^1U^8, G^{12}, G^{64}$     | $U^{52}$                          | $G^{51}, G^{74}, G^{10}, G^{22}, G^{18}, G^{69}, G^{68}, T^{4}, \phi^{55}N_3$ | $U^5, G^{67}, U^{29}, \phi^{55}N_1$ |
| $G^{73}$     | $G^7, s^1U^8, G^{12}, G^{24}$     | $U^{52}$                          | $G^{51}, G^{74}, G^{10}, G^{22}, G^{18}, G^{69}, G^{68}, T^{4}, \phi^{55}N_3$ | $U^5, G^{67}, U^{29}, \phi^{55}N_1$ |
| $U^{12}A^{23}$ | $s^1U^8, G^{24}, G^{42}$         | $U^{52}, \phi^{55}N_3, \phi^{55}N_1$ | $G^{51}, G^{74}, G^{10}, G^{22}, G^{18}, G^{69}, G^{68}, T^{4}, \phi^{55}N_3$ | $G^{74}, U^{29}$ |

**Fig. 6.** Imino proton titration studies of bovine tRNA$^{Trp}$ wild type and two mutants ($G^{73}$ and $U^{12}A^{23}$) with Mg$^{2+}$. The sample conditions are 10 mM sodium phosphate, 100 mM NaCl, pH 6.5 at a temperature of 30 °C. The binding curves of chemical shift versus Mg$^{2+}$ concentration for several typical imino protons are shown by fitting the data to a one- or two-binding-site model.
Previously it was shown that the discriminator base N73 is an important element on tRNA<sup>Trp</sup> toward the productive catalytic recognition by TrpRS with bacterial TrpRS preferring the presence of G73 and eukaryotic and archaeal TrpRS preferring the presence of A73 (21, 22). As shown in Fig. 8, mutation of A73 on wild type tRNA<sup>Trp</sup> to G73 brought about a steep decrease in tryptophanylation by human TrpRS, and mutation of G12C23 to U12A23 also brought about a significant decrease. The observation confirmed A73 as a major identity element and G12C23 as a minor identity element on bovine tRNA<sup>Trp</sup>.

**DISCUSSION**

Through 15N labeling in vivo with the use of different mutants to remove overlapping resonances, the resonances of all 26 observable imino protons participating in secondary and tertiary base pairings in bovine tRNA<sup>Trp</sup> hyperexpressed in E. coli have been assigned. By providing another example in addition to the earlier study of B. subtilis tRNA<sup>Trp</sup> (15), these findings establish the generality of this powerful experimental approach for characterization of tRNA structures by the combination of NMR and sequence mutagenesis. They also made possible an analysis of the relationship between Mg<sup>2+</sup> binding sites and tRNA conformation.

**Mg<sup>2+</sup> Binding Sites**—NMR spectroscopic studies of the wild type and mutant tRNA<sup>Trp</sup>, moreover, made possible a dissection of the Mg<sup>2+</sup> binding sites. Mg<sup>2+</sup> is known to strongly stabilize the native tertiary structures of tRNA even in the presence of substantial univalent salt concentrations (30), and a great deal of effort has been made to understand the Mg<sup>2+</sup> binding to tRNA.

The crystallographic structure of yeast tRNA<sup>Phe</sup>, in which coordinated magnesium ions were first identified, has provided a valuable basis for the thermodynamic analysis of Mg<sup>2+</sup> binding (31). Based on temperature factors (B factors) of the crystal structure, there are four strong Mg<sup>2+</sup> binding sites located on the D stem and D loop and six weaker sites distributed over the four stems and the anticodon loop. This suggests that the Mg<sup>2+</sup> binding depends on the specific localized structure on the tRNA, and the important question arises regarding the relationship between Mg<sup>2+</sup> binding and tRNA conformation. Since NMR is particularly useful for the investigation of macromolecular structures, in the present study NMR analysis of wild type and mutant tRNA<sup>Trp</sup> was performed to address this question.

Previously Mg<sup>2+</sup> binding to tRNA<sup>Gly</sup>, tRNA<sup>Fmet</sup>, tRNA<sup>Val</sup>, and mitochondrial tRNA<sup>Ser</sup> was investigated using 1H NMR spectroscopy (8, 32–35). Delineation of different Mg<sup>2+</sup> binding sites was limited, however, due to inadequate resolution and assignment of the imino protons in the 1D NMR spectra. In contrast, assignment of all 26 observable imino protons in the stems and tertiary base pairs of bovine tRNA<sup>Trp</sup> rendered straightforward the titration of chemical shift changes of individual imino protons as a function of Mg<sup>2+</sup> concentration. On this basis, in wild type tRNA<sup>Trp</sup> G7, sU8, G12, and G24 were found to be associated with strong Mg<sup>2+</sup> binding, U30 with medium binding, and 19 other protons with weak binding. Moreover, in so far that the Mg<sup>2+</sup> titration curves of the G7, sU8, G12, and G24 proton exhibited a clear maximum, their behavior requires fitting to a two-binding-site model rather than a one-binding-site model of Mg<sup>2+</sup> binding (Fig. 5 and Table II). The responses of imino protons to Mg<sup>2+</sup> addition in the G73 mutant tRNA<sup>Trp</sup> are closely similar to those observed in the wild type. The maximum chemical shift change induced by the addition of magnesium ions varies with the imino protons (Fig. 5). It is noteworthy that the maximum chemical shift change is not tightly correlated with the strength of Mg<sup>2+</sup> binding. For example, large chemical shift changes were displayed by sU51, U65, and G67 protons, and small chemical shift changes were displayed by G19 and U53, yet all five of these protons were associated with loose Mg<sup>2+</sup> binding. This is not entirely surprising. Since chemical shift changes are usually dependent on the extent of chemical environmental alteration and ring current variations associated with individual nucleotides (27, 36), these parameters are not expected to be correlated exactly with the thermodynamics of Mg<sup>2+</sup> binding.

Mutagenesis of the G12C23 base pair in the wild type to U12A23 altered the mode of Mg<sup>2+</sup> binding at the D stem and nearby region. Both sU36 and G24, affected by two molecules of Mg<sup>2+</sup> in the wild type, became associated with a single-site Mg<sup>2+</sup> binding mode (Fig. 6). Furthermore, the Mg<sup>2+</sup>-induced chemical shift change in sU36 in the mutant molecule over the higher Mg<sup>2+</sup> concentration above 2 mM was opposite in direction to the change in wild type. Such behavior indicates strongly the occurrence of important conformation change in the D stem region upon mutation of G12C23 to U12A23.

**Thermal Stability**—The binding of Mg<sup>2+</sup> to the various binding sites on tRNA<sup>Trp</sup> resulted in evident stabilization of both secondary and tertiary structures. In the absence of Mg<sup>2+</sup>, base pairings in the acceptor stem retained at 50 °C visible resonances even with all the tertiary interactions disrupted and the three other stems largely melted. This is to be expected given the seven base pairs, as many as five of these GC, in the acceptor stem. In the presence of 10 mM Mg<sup>2+</sup>, however, the 2D 15N-1H HSQC results suggest that the D stem was the most stable element in the molecule followed by T stem and acceptor stem with the anticodon stem having the lowest stability. Overall
tRNA<sup>Trp</sup> structure stability was increased by more than 15 °C upon the addition of 10 mM Mg<sup>2+</sup>. The exceptional stability of the D stem, with only four base pairs, in the presence of Mg<sup>2+</sup> is entirely consistent with the Mg<sup>2+</sup> binding sites delineated in Table II. Of the four nucleotide residues associated with strong Mg<sup>2+</sup> binding, G<sup>7</sup> is located on the acceptor stem next to the junction with D stem, while the s<sup>4</sup>U<sup>8</sup>A<sup>14</sup> base pair spans the D stem. G<sup>12</sup> and G<sup>24</sup> both form part of the D stem itself. This very special relationship between the D stem and strong Mg<sup>2+</sup> binding in all likelihood may be important rivets contributing to the remarkable stability of the D stem in the presence of 10 mM Mg<sup>2+</sup>.

As shown in Fig. 8, A<sup>73</sup> on bovine tRNA<sup>Trp</sup> functions as a major identity element, and G<sup>12C</sup><sup>23</sup> functions as a minor identity element. Mutation of these elements to G<sup>73</sup> and U<sup>12A</sup><sup>23</sup>, respectively, decreases tryptophanylation activity. Mutation of A<sup>73</sup> to G<sup>73</sup> brought about minimal alterations with respect to either NMR spectrum (Fig. 4) or Mg<sup>2+</sup> binding sites (Fig. 6). In contrast, mutation of G<sup>12C<sup>23</sup></sup> to U<sup>12A</sup><sup>23</sup> brought about evident alteration in the NMR spectrum (Fig. 4) as well as the Mg<sup>2+</sup> binding mode of s<sup>4</sup>U<sup>8</sup> and G<sup>24</sup>, attesting to the occurrence of conformational change in the U<sup>12A</sup><sup>23</sup> mutant molecule. Therefore A<sup>73</sup> as an identity element is most likely recognized directly by TrpRS. G<sup>12C<sup>23</sup></sup> as an identity element, on the other hand, evidently contributes to productive TrpRS-tRNA<sup>Trp</sup> recognition at least in part through the maintenance of an optimal conformation that was affected by mutation to U<sup>12A</sup><sup>23</sup>. The Mg<sup>2+</sup> binding sites mapped by NMR in this study are in agreement with the x-ray crystallography results (31) in that both point to the clustering of strong Mg<sup>2+</sup> binding sites in the D stem and its junctions with the acceptor stem and anticodon stem. Thus NMR analysis when combined with sequence mutagenesis has made possible extensive assignment of imino proton resonances, which in turn has permitted the mapping and characterization of multiple Mg<sup>2+</sup> sites on the tRNA. Once mapped and characterized, these sites provided an array of conformational markers that are very sensitive to conformational change in the tRNA molecule. This will open up the way for proving the relationship between tRNA sequence and conformation as well as the sequences and conformations optimal for recognition by cognate aminoacylation-tRNA synthetases.

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