Mercury(II) binding to s^4U in *E. coli* tRNA^{Val}

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ABSTRACT

The accessibility of the s^4U base in native tRNA^{Val} from *E. coli* was monitored by studying the binding of various mercurials. The relative binding order $HgBr_2 \sim HgCl_2 \gg CH_3HgOAc \sim CH_3HgCl \sim PCMB$ parallels approximately the steric requirements of linear HgX_2 or $RHgX$ compounds for S_N2 displacement by sulfur, although other factors are operative. Para-chloromercuribenzoate (PCMB) does not bind the thiolated nucleotide unless the tertiary structure of the tRNA is opened up by removal of Mg^{2+} ions and heating to 40° . Under these conditions, equilibrium dialysis measurements using ^{14}C -labeled PCMB showed one binding site ($n = 0.93$) with an association constant, K_1 , of $9 \times 10^4 M^{-1}$.

INTRODUCTION

We have recently reported² the strong binding of mercury(II) compounds to thiolated nucleosides. Reports of binding of mercury(II) containing reagents to the s^4U group found in several *E. coli* tRNAs have also appeared in the recent literature.²⁻⁵ The binding of p-chloromercuribenzoate⁶ to the s^4U base of *E. coli* tRNAs has been shown to occur only in the absence of magnesium ions.^{2,4} We have observed² the binding of mercuric chloride to *E. coli* tRNA^{Val} in the presence of Mg^{2+} ions at 25° , conditions under which PCMB exhibits no binding. If Mg^{2+} is removed, however, and the tRNA solution is heated to 40° , coordination of PCMB to the sulfur atom of s^4U occurs. Similar results have been observed⁴ for tRNA^{Phe} from *E. coli*. At 20° in the absence of Mg^{2+} ions, PCMB binding to tRNA^{Val} occurs, but to a lesser extent than at 40° . During the course of our study, the binding of pentafluorophenylmercury(II) chloride to s^4U in tRNA^{Tyr} in the presence of Mg^{2+} was reported.⁵ The relevance of this work to our own is discussed below.

In the present paper we describe equilibrium dialysis experiments that establish quantitatively the 1:1 nature of the PCMB- $s^{48}\text{U}$ interaction as well as the binding constant. Dialysis experiments also show that PCMB binding occurs to a greater extent in pure water solutions than in Tris buffer containing 0.1 M NaCl. The interaction of other HgX_2 and RHgX compounds with *E. coli* tRNA^{Val} has been studied from which it appears that size is an important, if not the sole, factor determining whether or not a given mercurial will bind tRNA in its "native" conformation (Mg^{2+} ions present, 0.1 M NaCl, 20°).^{8,9}

MATERIALS AND METHODS

Chemicals and Buffers. Unless otherwise stated, all experiments were carried out in 0.05 M Tris buffer solutions, pH 7.2, in the presence of 0.1 M NaCl; 0.01 M MgCl_2 was present or absent as indicated. Crystalline PHMB (PCMB when dissolved⁶) was purchased from Sigma Chemical Corp. The dihydrate form, $\text{NaO}_2\text{CC}_6\text{H}_4\text{HgOH} \cdot 2\text{H}_2\text{O}$ ($M = 397$), was established by (i) chemical analysis, (ii) a weight loss measurement of a sample dried at 100° over P_2O_5 , and (iii) measurement of the UV absorbance at 232 nm of a carefully weighed sample at pH 7 compared with the known molar extinction coefficient¹⁰ of 1.69×10^4 . Crystalline HgCl_2 (Baker Analyzed Reagent) was used without further purification as were Alfa Inorganics HgBr_2 (53% Hg by weight), CH_3HgOAc , and CH_3HgCl . ^{14}C -PCMB was purchased from Schwarz Bio-Research Inc. The specific activity determined by counting a known concentration (UV spectral criterion) was 12.5 C_1/mole . Dialysis tubing (Food Products Division, Union Carbide Corp.) was boiled twice in sodium bicarbonate solution, then in EDTA and SDS solutions, and finally was boiled in deionized water. For the equilibrium dialysis experiments the tubing was also soaked in Tris buffer solution for at least 1/2 day prior to use.

* tRNA Samples. Samples of tRNA^{Val} were gifts of A.D. Kelmers, Oak Ridge National Laboratory, and of Lars Rymo and Ulf Lagerkvist, Göteborg, Sweden. The Oak Ridge (OR) sample was claimed to have a valine acceptor activity of 1310 pmoles/ A_{260} unit and a terminal adenosine assay of 1590 pmoles/ A_{260} unit.

Studies in our laboratory and elsewhere (P. Cole, private communication) have shown a decrease in valine acceptor activity with time. Control experiments with mercuric chloride indicated the Oak Ridge and Swedish (S) samples to behave identically in their binding to the metal complex. The tRNA samples were initially dialyzed extensively against 0.05 M Tris buffer, pH 7.2, 0.01 M MgCl_2 , and 0.01 M NaCl to remove any impurities, such as sodium dithionite, that might interfere with metal binding. Solutions were stored frozen at -15° . To remove MgCl_2 , the tRNA solutions were dialyzed four times against 0.01 M EDTA, adjusted to pH ~ 6.4 with NaOH, then dialyzed three times against 0.05 M Tris buffer, pH 7.2, and 0.1 M NaCl. All dialyses were carried out at $\sim 5^\circ$.

Spectroscopic Measurements. Ultraviolet spectra were measured on a Cary 14 or Cary 15 spectrophotometer. Measurements at 40° were carried out in a thermostated cell. Aliquots (5 μl or more) of a solution containing a given mercury(II) complex were added to 2.5 ml of tRNA^{Val} solution in a 5cm pathlength cell. The tRNA concentration was calculated assuming a molecular weight for tRNA^{Val} of 2.5×10^4 and an absorbance (in 0.05 M Tris, 0.1 M NaCl, 0.01 M MgCl_2) of 20 at 260 nm for a solution of 1 mg/ml in a 1 cm pathlength cell.¹¹ Extinction coefficients reported for the s^{4U} chromophore were then obtained by dividing the absorbance measured at 338 (or 335) nm in a 5 cm cell by 5 times the tRNA concentration as determined from A_{260} . A 3% increase of A_{260} upon the removal of Mg^{2+} ions was taken into account. Circular dichroism measurements were performed on a Cary 60 spectropolarimeter equipped with a CD attachment. Measurements at 40° employed a thermostated CD cell.

Equilibrium Dialysis. The experiment was carried out by dialyzing approximately 5×10^{-3} μmoles of tRNA in 0.5 ml of 0.05 M Tris buffer solution containing 0.1 M NaCl and no Mg^{2+} ions against 3 ml of buffer solution containing from 0.04-0.23 μmoles of ^{14}C labeled PCMB. The dialyses, carried out in a water bath maintained at 40° , were complete in three hours as determined in a control without tRNA. Aliquots of solution taken both inside and outside the dialysis tubing were dissolved in Kinard's solution and their radioactivity was measured in a Packard liquid scintillation counter.

RESULTS AND DISCUSSION

Quantitative Study of the Binding of PCMB to s^4U of $tRNA^{Val}$.

Valyl tRNA from *E.coli* has a 4-thiouridine in the eighth position from the 5' end.¹² Its UV spectrum exhibits an absorption near 335 nm characteristic of thiolated nucleosides. At room temperature (ca 22-25°) in Tris buffer containing 0.1 M NaCl and 0.01 M $MgCl_2$ the absorption maximum is at 338 nm with measured extinction coefficients of $1.05 \pm 0.01 \times 10^4$ and $0.99 \pm 0.01 \times 10^4 \text{ M}^{-1} \text{ cm}^{-1}$ for the OR and S samples, respectively. In the absence of $MgCl_2$ and upon heating to 40°, the absorbance maximum shifts to 335 nm and increases by about 25% (Figure 1). Since the sodium ion concentration in the solution is 0.1 M (with additional cation present as Tris), the $tRNA^{Val}$ at 40° is assumed to be melted into the "cloverleaf" or "close variant" phase II form defined by Cole, Yang, and Crothers.⁸ When a metal compound is added which binds to the sulfur atom of the s^4U base, a spectral change such as that shown in Figure 2 occurs. By monitoring the disappearance of the 335 nm band as a function of added reagent, therefore, the binding of a given mercurial can be determined.

As reported earlier,² PCMB binds "phase II" but not native "phase I"⁸ $tRNA^{Val}$. A similar result was obtained in a study⁴ of ^{14}C -PCMB binding to $tRNA^{Phe}$. In the latter work, ^{14}C -PCMB was incubated with the tRNA at 45° in the absence of Mg^{2+} after which excess reagent was removed by dialysis against water. 1277 pmoles of ^{14}C -PCMB per A_{260} unit of $tRNA^{Phe}$ were incorporated. On the basis of a terminal adenosine assay of 1300 pmoles per A_{260} unit, binding was considered quantitative.⁴

An estimate of the binding of PCMB to $tRNA^{Val}$ was obtained in a similar assay in which 0.01 μ moles of tRNA in 0.5 ml Tris buffer (0.05 M, pH 7.2, 0.1 M NaCl) were incubated in the presence of 0.02 μ moles ^{14}C -PCMB for 1 hour at 40°. The solution was then cooled to room temperature and dialyzed twice against 250 ml of Tris buffer to remove excess PCMB. A measurement of the radioactivity showed that only 1500 pmoles of PCMB remained in the sample, indicating only 15% incorporation of PCMB.

Fig. 1, UV spectra of *E. coli* tRNA^{Val} (a) at 25° in the presence of 0.01 M MgCl₂; (b) at 40° in the absence of MgCl₂.

Fig. 2, UV spectra of *E. coli* tRNA^{Val} (a) at 25° in the presence of 0.01 M MgCl₂ (PCMB not bound); (b) at 40° in the absence of 0.01 M MgCl₂ (PCMB bound.)

To compare this result directly with that for tRNA^{Phe},⁴ the experiment was repeated only this time dialyzing against pure water. Under these conditions, 5000 pmoles (50%) of PCMB were incorporated (or 63% based on the terminal adenosine assay), somewhat less than that obtained for tRNA^{Phe}. It is apparent from these experiments that binding of PCMB to tRNA^{Val} occurs to a greater extent under conditions of low salt. Extensive dialysis against water would greatly reduce the salt concentration of the tRNA solution, resulting in a further opening up of the tertiary and/or secondary structure.^{8,9} This could allow greater binding of PCMB, either by making the s⁴U group even more accessible to the mercurial, or by exposing other binding sites for PCMB.¹³ Eventually, on prolonged dialysis, the PCMB concentration would itself diminish.

The method described above is clearly not a very satisfactory way to measure the extent of binding since dialysis against water continually reduces the free PCMB concentration, and thus the bound mercury, assuming equilibrium is maintained. Also, measuring the radioactivity remaining in the sample gives the total PCMB concentration, bound and free in solution, and is not an accurate measure of the amount bound. To obtain a quantitative measure of n , the number of binding sites, and K , the equilibrium constant, for PCMB and tRNA^{Val} under the same conditions as those used in the UV spectroscopic experiments, equilibrium dialysis using ¹⁴C-PCMB was performed as described under MATERIALS AND METHODS. Measurement of the radioactivity inside and outside the dialysis tubing after dialysis gave the total amount of bound and unbound PCMB in the tRNA solution at equilibrium. The quantity $r/[PCMB]_{\text{free}}$ versus r was plotted (Scatchard plot¹⁴), where r is the ratio $[PCMB]_{\text{bound}}:[tRNA]_{\text{total}}$. The plot will be linear for n binding sites with the same K and no electrostatic or other interactions among the sites.¹⁵ Since $r/[PCMB]_{\text{free}} = nK - rK$, n will be given by the intercept on the abscissa and K , by the intercept on the ordinate (nK) or from the slope ($-K$). From Figure 3 it is seen that there is only one binding site ($n = 0.93$) for PCMB on tRNA^{Val}, presumed to be the s⁴U, with $K = 9 \times 10^4 \text{ M}^{-1}$.

Fig. 3, Scatchard plot of the binding of ^{14}C -PCMB to *E.coli* tRNA^{Val} (40°, no Mg^{2+} present.) Data are plotted according to the equation $r/[PCMB]_{free} = nK - rK$ where r is defined in the text.

This binding constant is of similar magnitude to the equilibrium constants for the binding of phenylmercurials to other thiolated mononucleosides and polynucleotides. The equilibrium constants for the binding of PHMB to $s^6\text{Guo}$ and $s^8\text{Guo}$ have been measured² as $4.8 \pm 1.0 \times 10^5 \text{ M}^{-1}$ and $7.2 \pm 2.6 \times 10^6 \text{ M}^{-1}$, respectively. A constant of $1.8 \times 10^6 \text{ M}^{-1}$ has been reported¹⁶ for the binding of p-hydroxymercurybenzene sulfonate to $s^2\text{Urd}$ and $s^2\text{Cyd}$. Binding constants of $1 \times 10^6 \text{ M}^{-1}$ and $2.3 \times 10^5 \text{ M}^{-1}$, respectively, were found by equilibrium dialysis for the binding of ^{14}C -PCMB to poly($s^2\text{U}$) and poly($s^2\text{C}$).¹⁶ In each of these cases, spectrophotometric studies have shown that the mercury binds to the sulfur atom of the thiolated base.

The Binding of Other Mercurials to tRNA^{Val} . In the previous section the binding of PCMB to tRNA^{Val} at 40° in the absence of Mg^{2+} ions was reported. Cooling the solution to 20° does not result in any spectral change. When the solution is cooled to 20° and the Mg^{2+} ions added back, the intensity of the 338 nm band is fully restored, demonstrating that PCMB is extruded as the polymer returns to its native state. A likely explanation for this behavior is that, owing to the stereochemical requirements of the native tRNA, the PCMB is too large to fit in the space adjacent to the sulfur atom of the $s^4\text{U}$ base. X-ray structure results for yeast tRNA^{Phe} support this view.¹⁷ In the melted phase II form, however, the tertiary structure has opened up, permitting the formation of an Hg-S bond.

Displacement reactions of RHgX by various incoming ligands including sulfur donors are known to proceed by an $\text{S}_{\text{N}}2$ mechanism involving trigonally coordinated mercury as the activated complex.⁷ It is conceivable that the sulfur atom of the $s^4\text{U}$ in native tRNA^{Val} is sterically hindered and unable to form the trigonal intermediate (or transition state) required for bonding. In an attempt to probe the size of the pocket into which a given mercurial must fit in order to bind the $s^4\text{U}$ group of tRNA^{Val} , various mercury compounds were added to solutions of tRNA under native conditions (buffer, 0.01 M MgCl_2 , 25°). By monitoring the 338 nm band, the extent of binding was determined for HgCl_2 , HgBr_2 , $\text{CH}_3\text{Hg}(\text{O}_2\text{CCH}_3)$ (abbreviated CH_3HgOAc), CH_3HgCl ,

and PCMB. Figure 4 shows the plots of ϵ_{338} as a function of added mercury reagent (the OR and S samples had slightly different ϵ_{338} values) and the changes observed are summarized in Table 1. Similar titration curves for PCMB and HgCl_2 with modified forms of tRNA^{Val} (elevated temperature, removal of Mg^{2+}) are presented in Figure 5. From Figure 4 it is apparent that only HgCl_2 and HgBr_2 bind the $s^4\text{U}$ residue of native tRNA^{Val} . Neither methylmercuric chloride nor methylmercuric acetate show any significant degree of binding. Opening up the tRNA tertiary structure permits some PCMB binding at 20° in the absence of Mg^{2+} ions and increased binding, similar to that of the mercuric halides, at 40° in the absence of Mg^{2+} ions (Figure 5). Mercuric chloride also exhibits greater binding to phase II than to native tRNA^{Val} (Figures 4 and 5 and Table 1). A qualitative experiment showed that CH_3HgCl binds the $s^4\text{U}$ residue when the tRNA tertiary structure is modified.

None of the mercurials investigated is expected to dissociate in solution.^{18,19} Although mercuric chloride and mercuric bromide are present as the neutral dihalides (HgX_2) in pure water, they can form complex anions, HgX_3^- and HgX_4^{2-} , in solutions containing excess halide ion, X^- . The distribution of various halide complexes has been studied¹⁹ as a function of the $\text{X}/\text{Hg(II)}$ ratio by Raman spectroscopy for the $\text{HgCl}_2\text{-LiCl}$ and $\text{HgBr}_2\text{-LiBr}$ systems. For a solution that is 10^{-1} M in chloride ion, the species HgCl_2 , HgCl_3^- , and HgCl_4^{2-} are present in equimolar amounts whereas in solutions of 10^{-1} M bromide ion, the $\text{HgBr}_2\text{:HgBr}_3^-\text{:HgBr}_4^{2-}$ ratio is 1:4:3.^{19,20} All the experiments with tRNA^{Val} were carried out in solutions that were 0.1 M in NaCl . Thus the HgCl_3^- and HgCl_4^{2-} ions are present to a significant extent in solutions of HgCl_2 and, it seems safe to assume, an even larger proportion of mercury is present as $\text{HgBr}_2\text{Cl}_n^{n-}$ in the HgBr_2 solutions. There does not appear to be any evidence for anation reactions of RHgX compounds in the presence of chloride ion.²¹

From these considerations, and making the reasonable assumption that the neutral RHgX or HgX_2 mercurial reagent, rather than one of the anionic derivatives HgX_3^- , RHgX_2^- , etc., is the

Fig. 4, Titration of tRNA^{Val} at 338 nm, 25° , MgCl_2 present with (a) HgCl_2 (OR); (b) HgBr_2 (S); (c) PCMB (OR); (d) CH_3HgOAc (S); (e) CH_3HgCl (S), where (OR) and (S) indicate the source of the tRNA. Extinction coefficients have been adjusted for small volume changes.

Fig. 5, Titration of tRNA^{Val} with (a) PCMB at 335 nm, 40° , MgCl_2 absent (OR); (b) PCMB, at 338 nm, 20° , MgCl_2 absent (OR); (c) HgCl_2 at 335 nm, 40° , MgCl_2 absent (OR).

Table 1 Summary of tRNA^{Val} Spectral Changes Induced by Mercurial Reagents

Mercurial	[Hg] ²⁺ , M.	T, °C	No Hg(II)	Extinction Coefficient ^{a,b} [Hg(II)]/[tRNA] = 1	Maximum $\Delta \epsilon \times 10^{-4}$	% change	Fig. Ref.
HgCl ₂ ^a	0.01	25	1.06	0.72	0.45(3.9)	-0.61	58 4a
HgCl ₂ ^b	none	40	1.30	0.53	0.50(10)	-0.80	62 5c
HgBr ₂ ^a	0.01	25	0.99	0.48	0.23(9.8)	-0.76	77 4b
PCMB ^a	0.01	25	1.04	1.04	1.05(3.5)	~0	~0 4c
PCMB ^a	none	20	1.14	0.87	-----	(-0.27)	(24) 5b
PCMB ^b	none	40	1.30	0.74	0.63(2.7)	-0.67	52 5a
CH ₃ Hg(OAc) ^a	0.01	25	0.99	0.94	0.90(5)	-0.09	9 4d
CH ₃ HgCl ^a	0.01	25	0.99	0.93	0.90(5)	-0.09	9 4e

^a Molar extinction coefficient (ϵ) measured at 338 nm. ^b ϵ at 335 nm. ^c This column reports ϵ when the [Hg(II)]/[tRNA] ratio (in parentheses) is sufficiently large that ϵ is not changing significantly.

one that binds the s^4U base, the results of Table 1 can be rationalized in part on stereochemical grounds. The stereochemical accessibility of the various mercurial reagents for S_N2 displacement of X by sulfur can be measured by the relative sizes of their steric angles, defined in Figure 6.²² Values for these angles were computed from published mercury-ligand bond lengths²³ and van der Waals radii,²⁴ and are presented in Table 2.

Table 2 Steric Angles (θ) for Linear RHgX Molecules

Compound	Steric Angle (θ) ^a in Degrees
HgCl ₂	76
HgBr ₂	72
PCMB	63
CH ₃ HgCl	52
CH ₃ HgOAc ^b	31(52)

^a The steric angle θ is defined in Figure 6. Values were computed from known interatomic distances and van der Waals radii. ^b This compound is probably converted to CH₃HgCl in the buffer solutions used, thus the value in parentheses.

From the table it is apparent that the relative stereochemical accessibility (which varies directly with the steric angle) of the compounds studied here increases according to the series CH₃HgCl $\sim$ CH₃HgOAc < PCMB < HgCl₂ $\sim$ HgBr₂.

This calculation indicates that steric factors are at least partly responsible for the stronger binding (Table 1) of HgX₂ versus RHgX compounds to s^4U in native tRNA^{Val}. The complete inability of PCMB to bind suggests that the additional bulk of the phenyl ring might hinder its approach to the sulfur atom altogether. Other factors may also be considered to account for the measurably greater binding of the compounds HgBr₂ and HgCl₂. Perhaps the ability of the HgX₂ compounds to form two covalent linkages enables them to disrupt the tertiary structure in the region of s^4U base by binding to or cross-linking two of the normal bases, thus enhancing the reactivity of the s^4U residue. The binding of mercury salts to the bases of DNA has been well documented.¹³

Fig. 6, Steric angle (θ) for S_2 attack on a linear $YHgX$ molecule. The symbol a is the metal-ligand bond length, and r is the van der Waals radius. Values for θ can then be computed by geometry (Table II). See reference 23 for a related discussion.

The subtlety of factors involved in the binding of HgX_2 and $RHgX$ compounds to the s^4U base in *E. coli* tRNAs is further underscored by the report⁵ that pentafluorophenylmercuric chloride binds to both s^4U residues in $tRNA^{Tyr}$ in the presence of Mg^{2+} . A 10-15 fold excess of mercurial was added to achieve substantial binding, however. It is possible that binding the second s^4U (at position 9) in $tRNA^{Tyr}$ makes the first (at position 8) more accessible in the presence of Mg^{2+} ions by changing the tertiary structure. This explanation is consistent with the fact that the pentafluorophenylmercurated $tRNA^{Tyr}$ could not be aminoacylated unless the mercury was removed.⁵

The changes in the CD spectrum accompanying the various states of the tRNA are very small. The CD spectrum of $tRNA^{Val}$ at 25° in 0.05 M Tris, pH 7.2, 0.1 M NaCl, 0.01 M $MgCl_2$, has a small trough at 295 nm and a peak at 266 nm. This spectrum does not change upon addition of mercuric chloride in amounts up to a $[HgCl_2]/[tRNA]$ ratio of 20. When Mg^{2+} ions are removed by dialysis, the CD at either 25° or 40° retains the trough at 295 nm. At 40° , a small shift in the position of the maximum to 267 nm and a slight decrease in intensity ($5 \pm 2\%$) is observed. The binding of PCMB in the absence of Mg^{2+} ions causes a small further decrease ($\sim 2\%$) in the intensity of the maximum, but the trough at 295 nm remains. Addition of Mg^{2+} ions results in an

increase in the intensity of the 266 nm band. These results are substantially in agreement with those CD spectra reported in the literature.²⁵ In the presence of 0.1 M NaCl, a great deal of tertiary structure is retained. Even at 40°, the perturbation is not very large, implying that only a subtle change in the molecule is necessary to allow a mercurial that would not otherwise bind to react with the s⁴U group.

SUMMARY AND CONCLUSIONS

Under conditions of native tRNA^{Val} tertiary structure, i.e. with both 0.1 M NaCl and 0.01 M MgCl present, the relative binding of mercurials follows the order $\text{HgBr}_2 \sim \text{HgCl}_2 \gg \text{CH}_3\text{HgOAc} \sim \text{CH}_3\text{HgCl} \sim \text{PCMB}$, with the last three showing little if any binding unless Mg^{2+} ions are removed. This series can be crudely rationalized by stereochemical considerations, but other factors are probably operative. The binding of PCMB to phase II tRNA^{Val} has been shown to be quantitative and specific for the s⁴U residue. Mercury binding can be represented by the following scheme:

The loss of the proton on N(3) is postulated by analogy to the proton loss on N(3) observed by X-ray diffraction for 1-methyl-4-thiouracilyl-p-mercuribenzoic acid.²⁶ The selectivity of mercury binding to sulfur in thiolated polynucleotides offers the potential for probing specific sites on a macromolecule with heavy metal ions. Further studies toward this end are in progress.

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