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# Recognition and Interaction of Small Rings With the Ricin A-Chain Binding Site

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***ABSTRACT*** **Ricin A-chain is an N-glucosidase that attacks ribosomal RNA at a highly conserved adenine residue. Our recent crystallographic studies show that not only adenine and formycin, but also pterin-based rings can bind in the active site of ricin. For a better understanding of the means by which ricin recognizes adenine rings, the geometries and interaction energies were calculated for a number of complexes between ricin and tautomeric modifications of formycin, adenine, pterin, and guanine. These were studied by molecular mechanics, semi-empirical quantum mechanics, and ab initio quantum mechanical methods. The calculations indicate that the formycin ring binds better than adenine and pterin better than formycin, a result that is consistent with the crystallographic data. A tautomer of pterin that is not in the low energy form in either the gas phase or in aqueous solution has the best interaction with the enzyme. The net interaction energy, defined as the interaction energy calculated in vacuo between the receptor and an inhibitor minus the solvation energy of the inhibitor, provides a good prediction of the ability of the inhibitor to bind to the receptor. The results from experimental and molecular modeling work suggest that the ricin binding site is not flexible and may only recognize a limited range of adenine-like rings. Proteins 31:33–41, 1998.** © 1998 Wiley-Liss, Inc.†

**Key words: ricin structure; inhibitor design; energy minimization**

## INTRODUCTION

Ricin is a heterodimeric protein isolated from the seeds of the castor plant, *Ricinus communis*. The ricin toxin A-chain (RTA) is an N-glycosidase of 267 amino acid residues linked by a disulfide bond to the B-chain of 262 residues.[1] Ricin is an extraordinarily toxic molecule that attacks ribosomes by removing a specific adenine base from ribosomal RNA, thereby inhibiting protein synthesis.[2] The enzyme is exquisitely selective, targeting the first adenine base in the loop sequence GAGA, in a highly conserved region of rRNA. The x-ray crystal structure of ricin has been reported[3] and refined to 2.5 Å resolution.[4] In addition, the crystal structure of RTA, expressed from a cloned gene, has been solved to 2.3 Å resolution.[4] These x-ray studies support[5] a hypothetical RTA active site involving Tyr 80, Val81, Gly121, Tyr123, Glu177, and Arg180, and these assignments find support in protein engineering experiments.[7–9] Various possible ligands of RTA are shown in Figure 1. Monzingo and Robertus[10] carried out an x-ray analysis of formycin monophosphate (FMP), adenyl guanosine (ApG), and guanyl adenosine (GpA) in the active site of RTA, and found 1) that adenine, but not guanine, can bind in a pocket in the active site; and 2) that FMP binds more strongly than ApG. In the complex of FMP with RTA shown in Figure 2, the formycin ring system is sandwiched between the rings of Tyr80 and Tyr123 and there are at least five hydrogen bonds between the formycin ring and the RTA. The adenine ring of ApG binds in a similar fashion, although the crystallographic occupancy is lower.

There is considerable interest in identifying inhibitors of RTA. The protein has been widely used in the design of antitumor agents,[11] but has also been used by governments and terrorists as a poison.[12,13] Effective inhibitors might be useful to help control nonspecific toxicity in treatments with immunotoxins and could also serve as antidotes in poisonings. Schramm and coworkers synthesized a 14-mer ribonucleotide containing a stem of 5 bp and phenyliminoribitol at the position of the target adenine. This compound was a ricin inhibitor with $K_i = 0.7$ μM[14] and, while it is a promising start on inhibitor design, it is unlikely to have any practical application as a drug. We have examined instead smaller molecules as ricin inhibitors.



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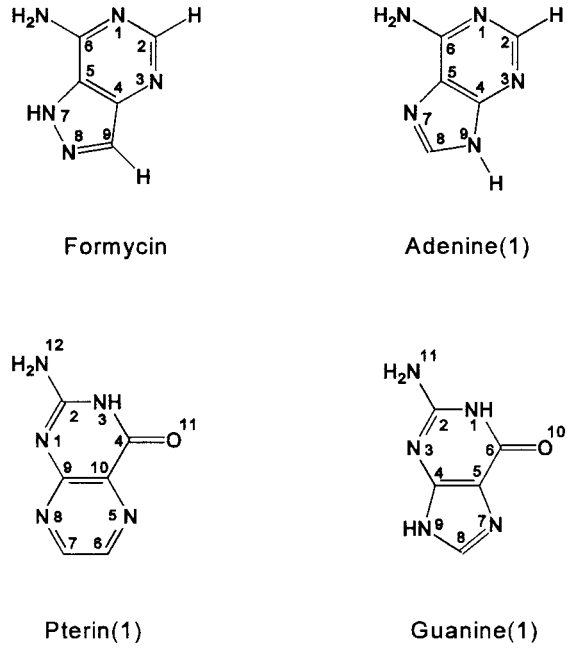


Fig. 1. RTA substrates. Nucleosides based on adenine, guanine, and formycin are linked to ribose or deoxyribose at position 9. Pterin derivatives discussed in this paper are modified at position 6.

Recently, we found that two pterin-based compounds, pteroic acid (PTA) and neopterin, behave as modest inhibitors of RTA and that the pterin ring can bind RTA more tightly than formycin or adenine.[15] The crystallographic occupancy of the pterin-based compounds in the RTA active site is about 100%, compared with about 50% for formycin and about 30% for the adenine in ApG.[10] Various small compounds that were also tested failed to act as ricin inhibitors and did not bind to RTA crystals. These compounds included AMP, 2-aminoformycin, phenyliminoribitol, imidazoleiminoribitol, p-nitrophenylriboamidrazone, emodin, 7-aminocephalosporanic acid, N-benzyloxycarbonyl-1-asparagine, 8-hydroxyguanine, 3-amino-4-hydroxybenzoic acid, 2'-O-anthraniloyladenosine-3',5'-cyclic monophosphate, folic acid, and pterin-6-carboxylic acid.

A simulation analysis of FMP binding in the active site of RTA has been reported[16] but there has been no systematic modeling study of the binding modes and interaction energy between RTA and compounds containing small rings. Thus, to aid in the identification of RTA inhibitors, we decided to seek a correlation between binding behavior and binding energies of formycin, adenine, pterin, and guanine and to see if computational methods could predict ligand binding to RTA in a qualitative or semi-quantitative sense. The results of this investigation are the subject of this paper.

## MATERIALS AND METHODS

All molecular mechanics operations were carried out using the Tripos force field (SYBYL software, v. 6.1[17]) running on a Silicon Graphics Workstation. The coordinates of the entire 267 residue RTA molecule, containing a given ligand, were derived from x-ray analysis and the structure was minimized in this force field. Energy minimizations were carried out with the Powell algorithm as implemented in SYBYL, terminating when a 0.005 energy gradient shift was obtained. A distance-dependent constant function dielectric constant of 1.5 was used to compute electrostatic effects to simulate the dielectric internally in the protein and the effect of the solvent on the protein. A non-bonded cutoff of 8 Å was used. The net atomic charges used for the RTA residues were calculated by the Kollman method,[18] which gave the best results when compared with those derived from ab initio calculations using the 6-31g***s basis set. The atomic charges in the ligands were derived from full ab initio minimizations conducted using the 6-31g** basis set.

In the energy minimization protocol, the crystal complexes of RTA-FMP[10] and RTA-PTA[15] were minimized and then the substrates were replaced by formycin, adenine, pterin, or guanine, and the new complexes were re-minimized in the same way as before. The interaction energy in these new energy-minimized complexes was calculated by subtracting the energy of the free ricin and of the uncomplexed ligand (in its binding conformation) from the energy of the complex. The solvation energy of the substrate in aqueous solution was calculated using AMSOL with AM1-SM2 Hamiltonians[19,20] and the net interaction energy was calculated as the interaction energy minus the solvation energy. This program has been found[19,20] to give fairly accurate estimates, normally within 1 kcal/mol of the experimental data, of the solvation energy for small molecules. The various x-ray analyses of RTA complexes suggest that the enzyme conformation is unlikely to undergo major changes as a result of the binding process. The ligands studied are also all rigid molecules and because of this it was assumed that the entropy of binding was essentially the same in each case and that molecular dynamics simulations would shed little light on the problem.

## RESULTS AND DISCUSSION

The hydridization of an amino nitrogen atom connected to an aromatic ring depends on the environment. The deviation from the ring plane of the hydrogens attached to such nitrogens is given in Table 1, in which it can be seen that some amino nitrogens, such as those in adenine(1) or pterin(2) are essentially $sp^2$hybridized; others, such as those in formycin or guanine, have both $sp^2$ and $sp^3$ characters. If the sum of the deviations from the ring

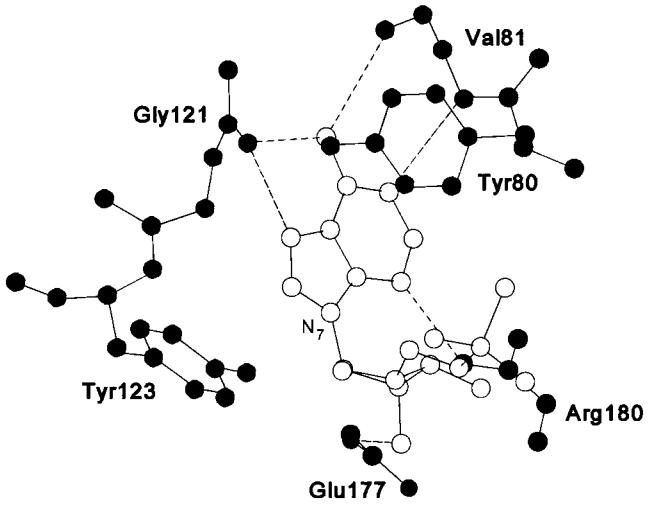


Fig. 2. The crystallographically observed complex of RTA and FMP. The structure was determined by Monzingo and Robertus[10]. The figure shows the substrate recognition cleft occupied by FMP, an AMP analog. Hydrogen bonds are shown as dashed lines.

**TABLE I. Sum of the Deviations From the Plane of the Aromatic Ring of the N-H Hydrogens According to Ab Initio (6–31 g**) Minimization**

| Structure | Deviation (°) |
|---|---|
| Formycin | 41.1 |
| Adenine (1) | 0.7 |
| Adenine (2) | 44.0 |
| Pterin (1) | 37.1 |
| Pterin (2) | 0.8 |
| Pterin (3) | 36.9 |
| Pterin (4, ion) | 0.1 |
| Guanine (1) | 38.1 |
| Guanine (2) | 27.8 |
| Guanine (3) | 32.1 |
| Guanine (4) | 41.3 |

plane of both hydrogens exceeded 30°, the nitrogen was treated as $sp^3$ hybridized in subsequent molecular mechanics calculations.

The ligands examined in this study are shown in Figure 3. The net atomic charges, as calculated by ab initio minimization with the 6-31g** basis set, are given in the figure. The molecular energies of the ligands in the gas phase, calculated using the 6-31g** basis set, are listed in Table 2, which also contains the free energies of the ligands in the aqueous phase, the solvation energies of the ligands, both of which were calculated by the AMSOL program.Table 2 also contains the energies of RTA-ligand interactions, calculated using the Sybyl molecular mechanics package and partitioned into van der Waals and electrostatic components. The last column in Table 2 provides the net interaction energy.

### Formycin

FMP is an analog of AMP, the natural substrate for RTA-mediated depurination. The binding of FMP to ricin has been examined crystallographically[10,21] and computationally[15,16] and the interaction between RTA and singly charged FMP was found to be -89 kcal/mol.[15] There is only a single important tautomer of the formycin base, and this should be the most stable structure both in aqueous solution and in the ricin binding site. In the energy-minimized formycin-RTA complex, the formycin forms seven hydrogen bonds to the enzyme with an interaction energy estimated at -43.7 kcal/mol. In this model, shown in Figure 4, the orientation of the formycin is generally similar to that observed crystallographically in the RTA-FMP complex, which is shown in Figure 2, but in the free formycin case, the ring system is shifted slightly to permit formation of an additional hydrogen bond, between $N_8$ of the formycin and the backbone NH of Tyr123. In addition, because the ligand lacks a sugar ring, the guanidinium of Arg180 reorients itself so as to participate in two hydrogen bonds, rather than one, to $N_3$ of the formycin.

### Adenine

Adenine is a base in the natural substrate for RTA and is specifically recognized by the enzyme, at least when it is part of the GAGA loop context. There are two major tautomers of adenine, as shown in Figure 3, but adenosine nucleotides are derivatized at $N_9$ and so are frozen into the adenine (1) tautomer. The binding of adenine (1) to RTA is shown in Figure 5 and the energy data in Table 2 suggest that free adenine (2) should interact more strongly with RTA than does adenine (1); a difference of about 1.6 kcal/mol in favor of adenine (2) is calculated. The main reason for this is that in adenine (2) as shown in Figure 5, the hydrogen on $N_7$ can form a hydrogen bond to the backbone oxygen of Gly121, while adenine (1) has no hydrogen on $N_7$. Weston et al.[22] have observed that AMP is hydrolyzed in a tetragonal form of crystalline RTA leaving only adenine bound. The occupancy was low, however, and it was not

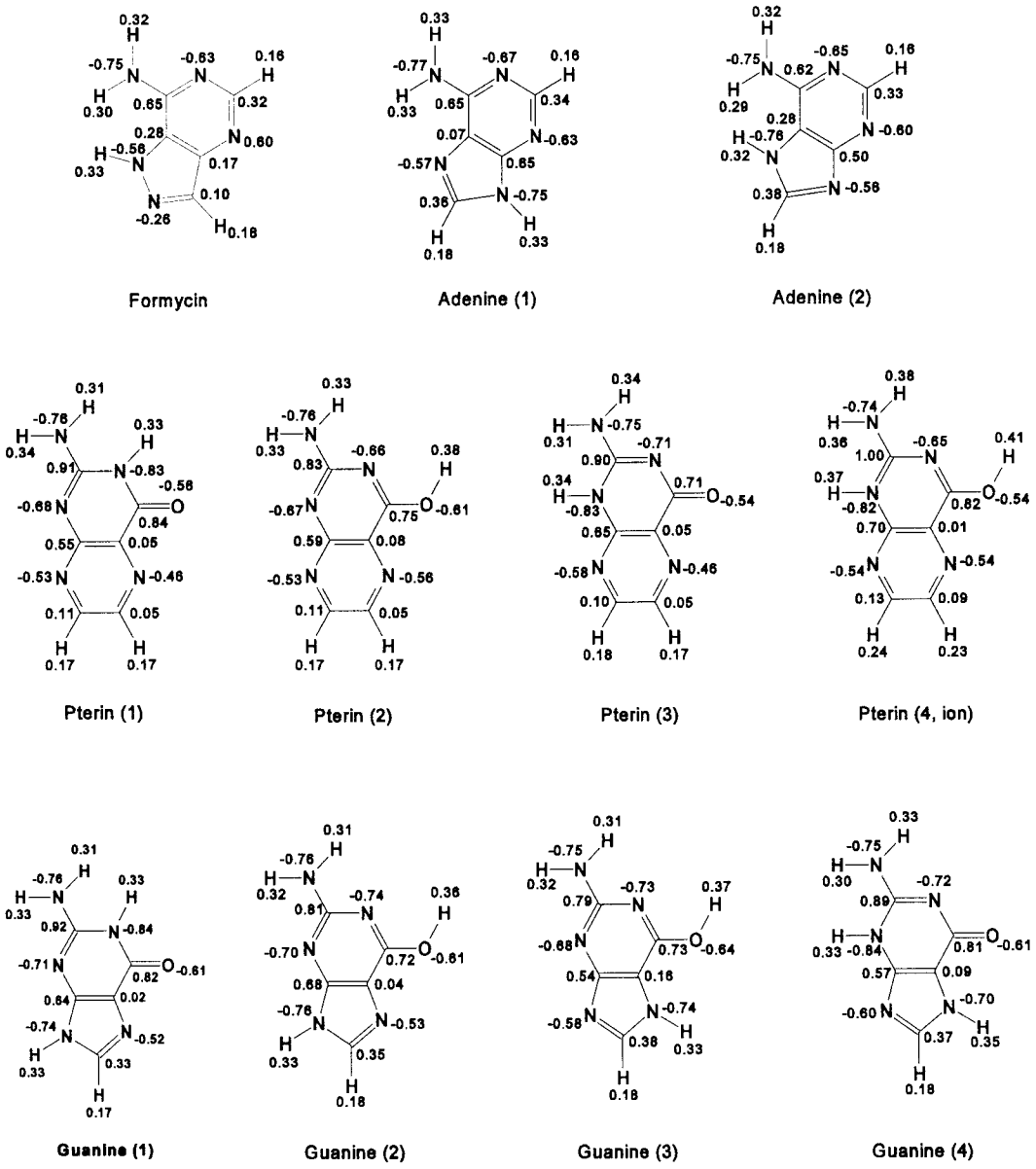


Fig. 3. Partial charges in compounds examined in this study. Charges were derived from full ab initio minimization and used in subsequent docking experiments with RTA.

possible to determine the tautomeric form of the adenine. In the design of inhibitors, derivatives of either form should be equivalent.

## Pterin

PTA has been shown to be an inhibitor of RTA and pterins are thus a worthwhile subject for inhibitor studies. Pterins have in principle, the four tautomeric forms shown in Figure 3, and the x-ray structures of the PTA-RTA and neopterin-RTA complexes suggest that in the RTA binding site, pterin (3) is the preferred form. This is confirmed by the calculated net interaction energy of -28.2 kcal/mol (Table 2). The minimized interaction energies between RTA and each of tautomers of pterin are give in Table 2; the complex formed between RTA and pterin (1) is shown in Figure 6. Pterin (1) has an interaction energy with RTA of -21.5 kcal/mol, comparable to that of formycin (-22.9 kcal/mol). The $N_{12}$ amino substituent at $C_2$ forms hydrogen bonds to the backbone oxygens of Gly121 and Val81, reminiscent of the behavior of the primary amino group in both formycin (Fig. 4) and adenine (Fig. 5). The oxygen of the $C_4$ carbonyl ($O_{11}$ in Fig. 1) forms hydrogen bonds to the backbone NH of Val81 and to the hydroxyl oxygen of Ser176, while $N_8$ is hydrogen bonded to the

**TABLE II. Molecular Energy (kcal/mol) From Ab Initio and Molecular Modeling Calculations of Different Species in Uncomplexed, Solvated Form and Bound to Ricin A Chain**

| Compound | Gas phase energy | Aqueous phase energy | Solvation energy (SE) | RTA interaction energy | | | |
|---|---|---|---|---|---|---|---|
| | | | | vdW | Elec. | Total (IE) | Net (IE-SE) |
| Formycin | −291,466.8 | 84.6 | −20.8 | −19.2 | −24.5 | −43.7 | −22.9 |
| Adenine (1) | −291,501.1 | 63.7 | −23.1 | −18.0 | −19.4 | −37.4 | −14.3 |
| Adenine (2) | −291,491.6 | 69.9 | −23.6 | −19.0 | −20.5 | −39.5 | −15.9 |
| Pterin (1) | −362,244.0 | 26.6 | −23.6 | −23.1 | −22.0 | −45.1 | −21.5 |
| Pterin (2) | −362,242.9 | 33.4 | −21.8 | −22.8 | −21.8 | −44.6 | −22.8 |
| Pterin (3) | −362,238.0 | 30.5 | −25.2 | −226. | −31.8 | −53.4 | −28.2 |
| Pterin (4, ion) | −362,485.9 | −62.4 | −75.0 | −22.9 | −24.6 | −47.5 | 27.5 |
| Guanine (1) | −338,486.8 | 22.1 | −26.8 | −18.7 | −16.1 | −34.8 | −8.0 |
| Guanine (2) | −338,487.0 | 27.4 | −22.1 | −21.8 | −20.0 | −40.8 | −18.7 |
| Guanine (3) | −338,482.4 | 30.0 | −25.3 | −20.8 | −33.0 | −53.8 | −28.5 |

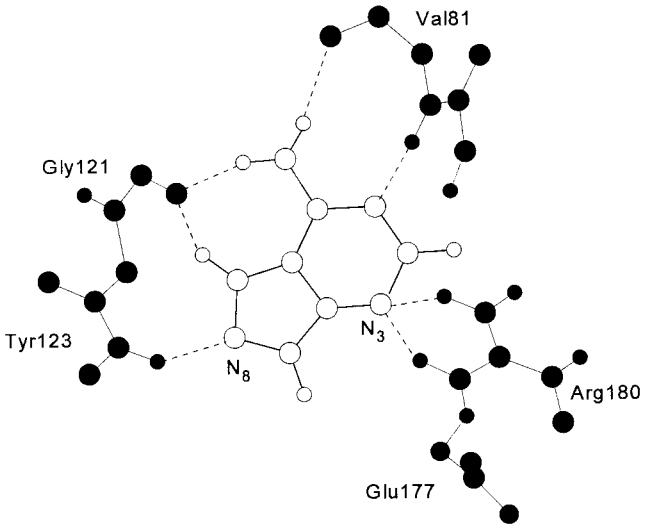


Fig. 4. Interactions between RTA and formycin.

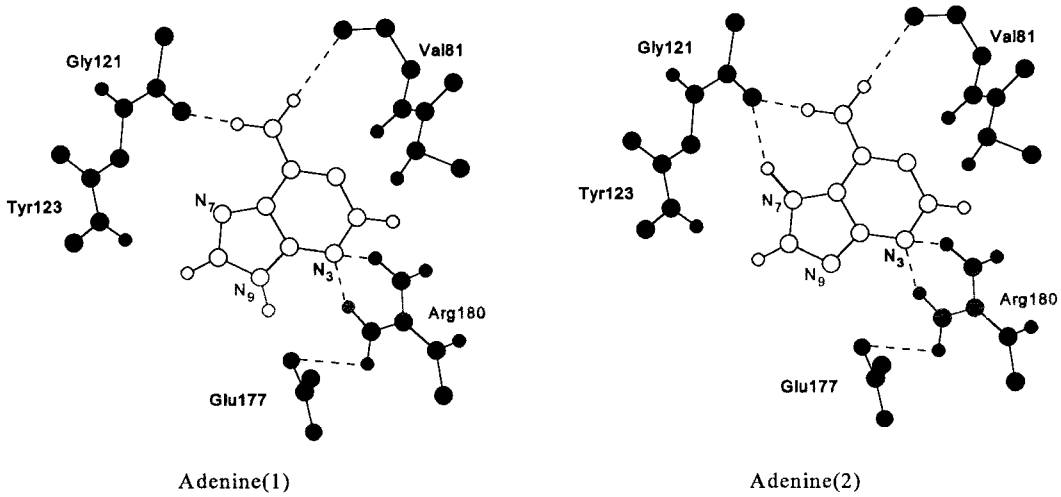


Fig. 5. Interactions between RTA and adenine tautomers.

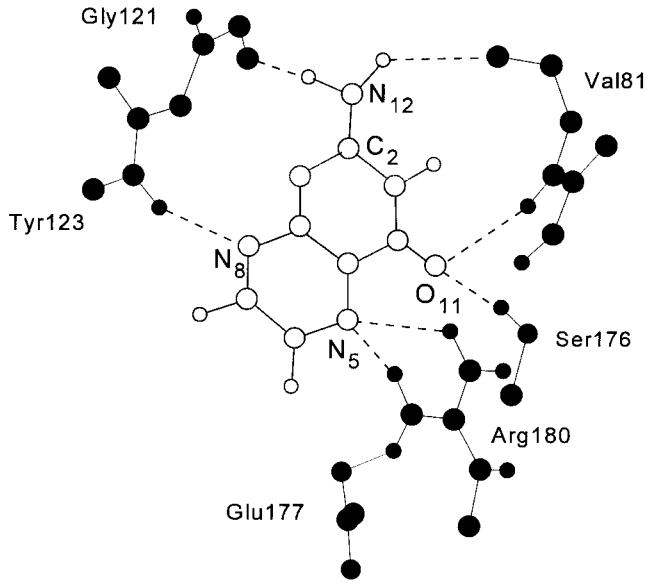


Fig. 6. Interactions between RTA and pterin (1) tautomer.

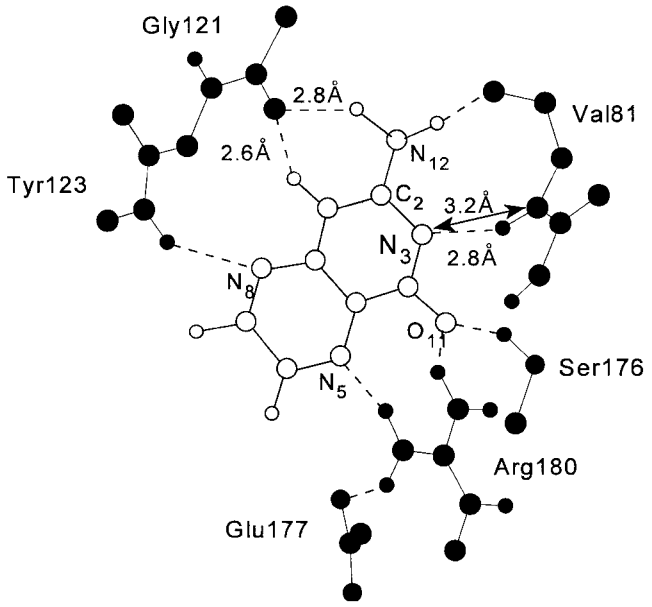


Fig. 7. Interactions between RTA and pterin (3) tautomer.

backbone NH of Tyr123 and $N_5$ forms two hydrogen bonds to Arg180. The interaction with RTA is as strong for pterin (1) as for formycin and suggests that pterin (1) can bind effectively to RTA. Pterin (3), however, should bind even more strongly to judge by its hydrogen bonding pattern, which is shown in Figure 7. This shows that pterin (3) forms all the hydrogen bonds formed by pterin (1) and in addition, has one additional hydrogen bond, between the hydrogen at $N_1$ and the backbone carbonyl oxygen of the Gly121 of RTA. A comparison of the RTA binding of pterin (1) and pterin (3) is shown in Figure 8, in which strong hydrogen bonds are shown as dashed lines and weaker hydrogen bonds as dotted lines. The pterin (1) complex has three strong and four weak hydrogen bonds while the pterin (3) complex has six strong and two weak hydrogen bonds. In particular, the absence of a hydrogen on $N_3$ of pterin (3) allows formation of a 2.8 Å hydrogen bond from that nitrogen to the backbone NH of Val81 and the hydrogen on $N_1$ of pterin (3) forms a hydrogen bond to the backbone oxygen of Gly121. As a result primarily of these two new hydrogen bonds, the interaction energy for pterin (3) is -28.2 kcal/mol, and the complex is 6.7 kcal/mol more stable than that formed by pterin (1). X-ray data support this conclusion because the measured distance between $N_3$ of the pterin and the backbone N of Val81 is 3.2 Å —impossible if both these nitrogens carry hydrogen, as in pterin (1). Furthermore, the distances between

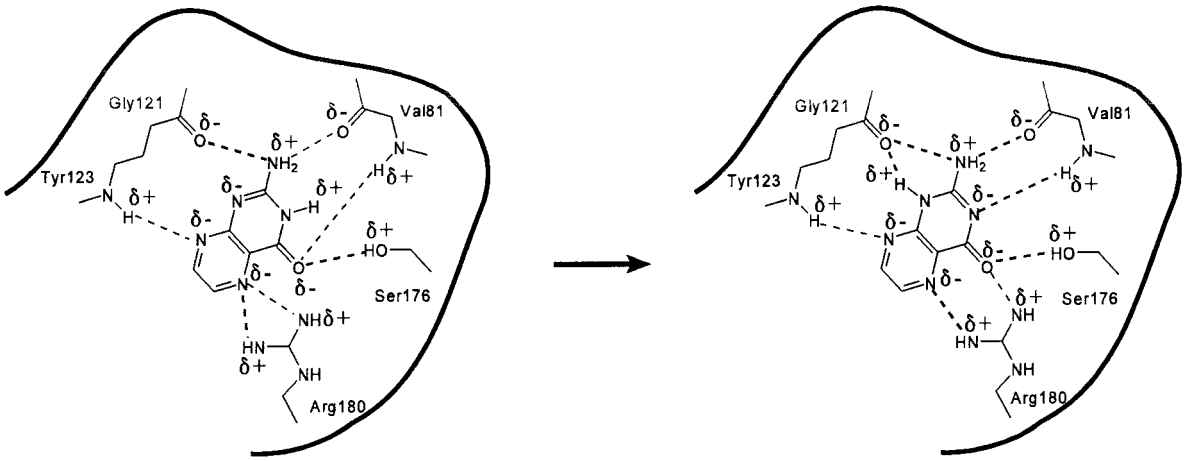


Fig. 8. Interactions between RTA and two tautomers of pterin: left, pterin (1); right, pterin (3). Strong hydrogen bonds (2.6–2.8 Å) are denoted by heavy dotted lines and weaker hydrogen bonds (3.0–3.2 Å) by lighter dotted lines.

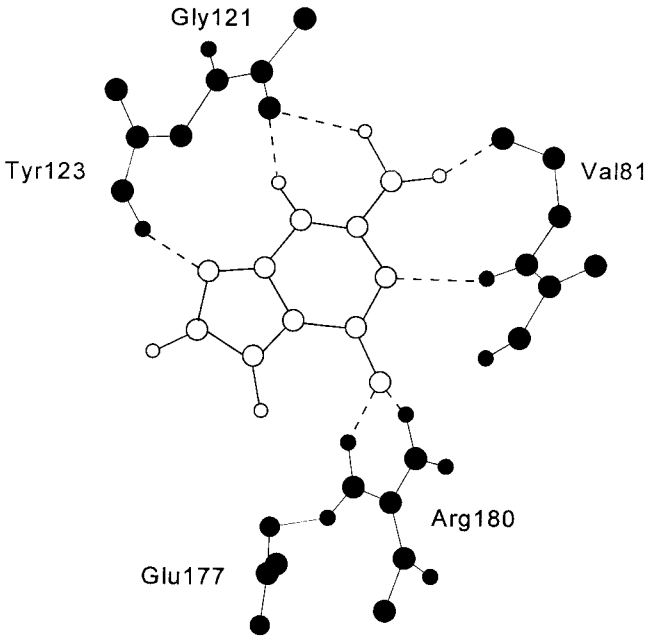


Fig. 9. Interactions between RTA and guanine.

the oxygen of Gly121 and $N_1$ or $N_{12}$ of the pterin are 2.6 Å and 2.8 Å respectively, which means that these two hydrogens bonds are strong and this adds to the stability of the RTA-pterin (3) complex.

The mode of binding of pterin (3) to RTA may follow from simple equilibrium considerations. Pterin (3) is less abundant in solution than its more stable tautomer, pterin (1), but once bound, the equilibrium may shift to produce more pterin (3). Alternatively, it may be that pterin (1) can recognize the RTA active site and during or after binding, a shift of protons from $N_3$ to $N_1$ takes place, generating the pterin (3) structure. This process is illustrated in Figure 8. The tautomerization may be triggered by the repulsive forces between the positive charges on Arg180, Ser176, and Val81, and the positive charge on the -NH- at $N_3$ of pterin (1). At the same time, there is an incentive for protons to move to $N_1$, which is surrounded by negative charges on Gly121 and Val81.

The strong binding of RTA to PTA is due to the binding to the pterin (3) ring system, which is further augmented by interactions with the benzoate-containing group at $C_6$ of the pterin. The benzoate ring makes van der Waals contact with Tyr80 and the benzoate carboxyl group forms a hydrogen bond with Asn78. Consistent with this observed binding, our earlier calculations[15] suggested that the interaction energy between RTA and PTA is -106 kcal/mol.

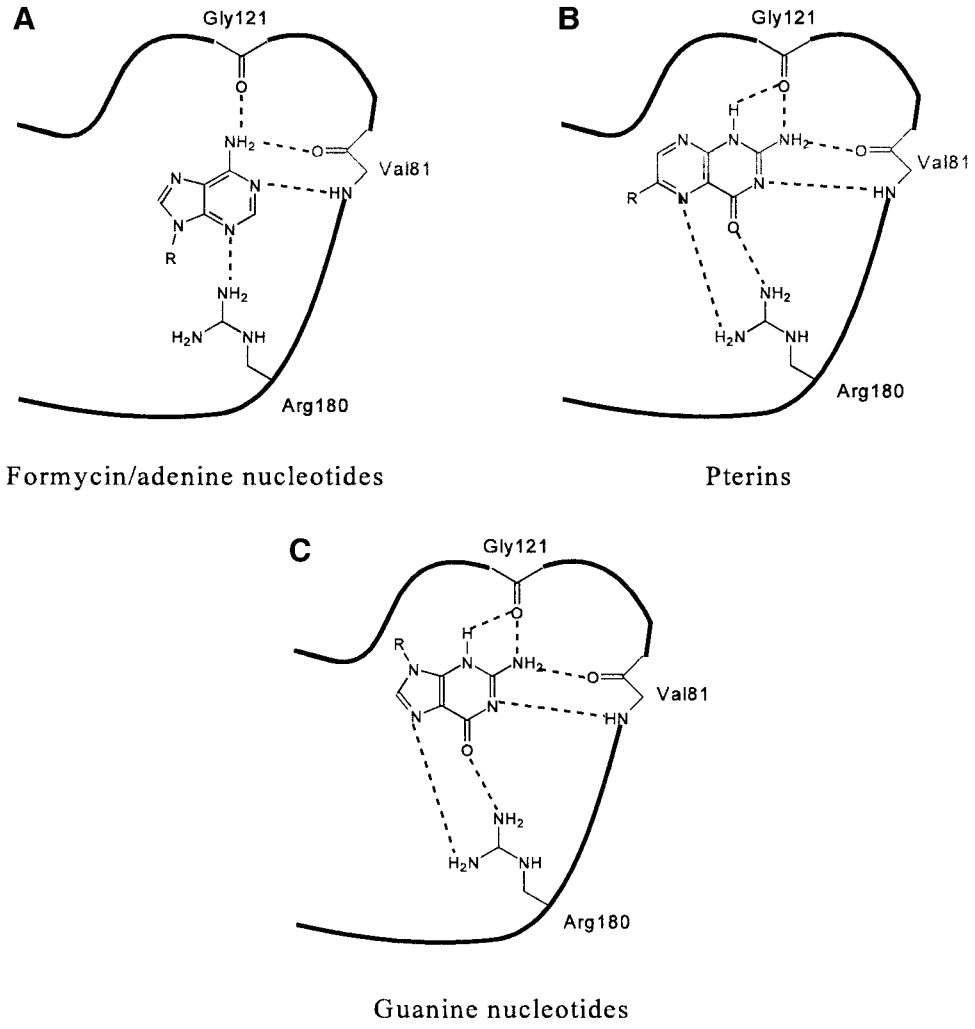


Fig. 10. Correlation of ligand structure for the design of RTA inhibitors. The binding to RTA of ligands based on formycin/adenine (**A**), pterin (**B**), and guanine (**C**) is shown.

The orientation of the pterin ring system in the binding site is slightly different for PTA compared with free pterin. The latter forms a hydrogen bond between its carbonyl oxygen at $C_4$ and the hydroxyl oxygen of Ser176 while this hydrogen bond, if it forms at all, is much weaker (3.4 Å) in the PTA-RTA complex. It may be possible, however, to exploit Ser176 in the design of RTA inhibitors.

**Guanine**

Guanine derivatives have many of the features of pterins and thus the binding to RTA of guanines is of interest. Guanine exists in four tautomeric modifications (Fig. 3) and ab initio calculations suggest that guanine (1) is the most stable tautomer, while guanine (4) is the least stable. The data in Table 2 show that guanine (4) interacts most strongly with RTA (-28.5 kcals/mol) and a model of this complex is shown in Figure 9. Two hydrogen bonds are formed between the carbonyl oxygen of guanine (4) and Arg180 and other atoms in the guanine interact with the RTA just as in the pterin (3) case. The stability of the RTA-guanine (4) complex, compared with that with guanine (1), arises from the same considerations as have been described for pterin (3) binding compared with that of pterin (1). The interaction energies presented in Table 2 had suggested that the guanines may be useful inhibitor candidates, but attempts to bind 2-amino-8-hydroxyguanine to crystals of RTA were unsuccessful, perhaps due to the poor solubility of this guanine derivative.

Calculations suggest that the interaction energies of the guanines with RTA are high relative to those of the adenines and a question arises as to why the RTA binding is specific to adenine residues, although guanine residues are present. Careful examination of the models shows that guanine cannot bind to the RTA active site for simple steric reasons. The first panel of Figure 10 is a view, derived from crystallographic measurements, of the binding to RTA of adenine-containing nucleotides such as FMP and ApG. Figure 10B shows the observed binding of the

pterin-based ligands, and Figure 10C depicts a guanine (4) derivative. It can be seen that the guanine (4) can adopt an electronic configuration similar to that of the pterin in Figure 10B, but its orientation is almost diametrically opposite to that of the adenine in Figure 10A. Because of this, the ribose attached to the guanine will collide with the protein structure, preventing binding. Non-nucleosidic guanine derivatives may still be expected to bind well to RTA if they are modified at positions 8 or 9. It should be recalled, however, that 2-amino-8-hydroxyguanine failed to bind to RTA, and it is possible that the interaction energy of guanine (1), calculated at -8.0 kcal/mol, is simply too low compared with that of pterin (3) (-28.2 kcal/mol) with the result that initial recognition and binding of the guanine is unlikely.

## CONCLUSIONS

The active site of RTA involves the backbones of RTA residues Gly121, Asn122, Tyr123, and Val81 and the side chains of Arg180, Tyr80, Tyr123, Glu177, and Trp211. The interaction energy from hydrogen bonding is a major determinant of RTA substrate specificity, in particular, the recognition by RTA of a specific adenine base in a particular rRNA context. The main hydrogen bonding sites in the RTA are the carbonyl oxygens of Gly121 and Val81, the amide nitrogen of Val81, and the side chain of Arg180. The carbonyl groups and backbone amides are rigid, as is the side chain of Arg180, which forms an aromatic stack with Trp213[3] and which is strongly immobilized by hydrogen bonds to the carbonyl of Glu177 and the amide carbonyl oxygen of Asn78. The Arg side chain can move only within a very limited space and always maintained its geometry during energy minimizations.

All these findings suggest that RTA has evolved to include a fairly rigid receptor that binds specific, complementary ring structures that necessarily are very similar to that of adenine. It is possible for compounds such as pterins to bind to RTA; compared with adenine, they make additional contacts and have stronger interaction energies with RTA. Clearly, these are important considerations in inhibitor design but it may be that even ligands that interact with every binding point in the active site will not be strong inhibitors and that additional groups outside the adenine recognition site will have to be exploited in the design of potent RTA inhibitors. The RTA has evolved to recognize an extended rRNA substrate and its strong binding to ribosomes ($K_m$ about 1 μM) arises partly from interactions at sites remote from the catalytic adenine recognition site. It may be necessary to target such areas for the design of optimum RTA inhibitors.

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