RNA (1996), 2:395–403. Cambridge University Press. Printed in the USA.

REVIEW

Structure and function of the hammerhead ribozyme: An unfinished story

DAVID B. MCKAY

Beckman Laboratories for Structural Biology, Department of Structural Biology, Stanford University School of Medicine, Stanford, California 94305-5400, USA

INTRODUCTION

Two crystallographic structures have been reported recently for the hammerhead ribozyme (Pley et al., 1994; Scott et al., 1995). Although the constructs were quite different (one was an RNA–DNA complex; the other was an all-RNA complex with a 2′-methoxy-2′-deoxyribose at the active site to prevent bond cleavage), as were the crystal stabilization conditions (high Li_2SO_4 versus low ionic strength), the structures are nearly identical in tertiary fold and conformation; the primary structural difference arises from the presence of a 2′-hydroxyl on the nucleotide 5′ to the cleavage site in the all-RNA structure ($U_{16.1}$), which forms specific hydrogen bonds that are not present in the RNA–DNA complex. The ribozyme is Y-shaped in the crystals, with stems I and II forming the arms of the Y and stem III making the base, in agreement with fluorescence energy transfer (Tuschl et al., 1994) and electrophoresis (Amiri & Hagerman, 1994; Bassi et al., 1995) data demonstrating the proximity of stems I and II and the near-collinearity of stems II and III in solution. The catalytic "core" of the ribozyme has two structural domains. The consensus CUGA sequence (nucleotide positions C_3–A_6) forms a sharp turn that is identical in conformation to the uridine turns observed originally in tRNAs (Quigley & Rich, 1976). The remainder of the core forms a non-Watson–Crick duplex that includes a tandem GA mismatch; the statistically greater-than-random frequency of tandem GA mismatches in sequences of larger RNAs leads one to wonder whether this is a structural motif that will appear elsewhere (Gautheret et al., 1994). The simplicity of the structure belies its ability to function as an enzyme.

Much to the dismay of at least some of the participants in the crystallographic endeavors, neither structure is in a "catalytic" conformation. The hammerhead ribozyme cleaves a phosphodiester bond to give a 2′,3′-cyclic phosphate and a 5′ hydroxyl as products in an Mg^{2+}-dependent reaction (Buzayan et al., 1986; Hutchins et al., 1986). The 2′ hydroxyl of the ribose 5′ to the scissile bond (hereafter referred to as "at the cleavage site") is absolutely required for activity (Dahm & Uhlenbeck, 1990), the *pro*-R_P nonbonded oxygen of the phosphate has been identified as a site of ligation of a catalytic divalent ion (Koizumi & Ohtsuka, 1991; Slim & Gait, 1991), and the pH dependence of the bond cleavage rate favors a model in which a hydroxide ion bound to the catalytic divalent ion abstracts a proton from the 2′ hydroxyl (Dahm & Uhlenbeck, 1991; Dahm et al., 1993). The configuration of the phosphate is inverted by the reaction (van Tol et al., 1990; Koizumi & Ohtsuka, 1991; Slim & Gait, 1991), arguing strongly that bond cleavage proceeds with in-line attack by the 2′ hydroxyl on the scissile phosphorus-oxygen bond; however, in the crystallographic structures, the active site is not in a conformation that would allow in-line attack. It is apparent that, at a minimum, substantial twisting at the active site would be required to align the major players for catalysis (with either the nucleotide flipping outward from the ribozyme, or the phosphate twisting inward—described in detail in Pley et al. [1994]); a specific proposal for rearrangement at the cleavage site, based on the structure of the all-RNA hammerhead, has been presented (described in detail in Scott et al. [1995]). Further, there seems to be no plausible alternative chemical scheme that can be invoked to circumvent this dilemma. Adjacent attack by the 2′ hydroxyl seems improbable because the 2′ oxygen is >3.5 Å from the target phosphorus atom; additionally, this would not lead to inversion of configuration of the phosphate. Adding a tandem step of mutarotation after adjacent attack to circumvent the stereochemistry difficulty creates a somewhat complex catalytic scenario of phosphodiester cleavage, for which there are few, if any, established precedents.

In such a context, some discretion must be exercised in evaluating the pertinence of the crystallographic structures to understanding the catalytic mechanism of

Reprint requests to: David B. McKay, Beckman Laboratories for Structural Biology, Department of Structural Biology, Stanford University School of Medicine, Stanford, California 94305-5400, USA; e-mail: mckay@cellbio.stanford.edu.

the ribozyme. It is fortunate that two independent hammerhead structures that show only minor differences are available, because in concert they argue strongly that the structures are valid, that they are not fraught with artifactual information due to the use of an RNA–DNA complex or particular crystallization conditions, and that they must have some relation to the overall catalytic cycle.

The kinetic scheme of a hammerhead has been elucidated in detail (Hertel et al., 1994; Hertel & Uhlenbeck, 1995). The activation energy for bond cleavage is ~22 kcal/mol, and the bond cleavage rate is ~1 min^{-1} at 25 °C. The equilibrium of the reversible cleavage/ligation reaction is ~10^2 in favor of cleavage; bond cleavage results in a substantial increase in entropy, suggesting that the product is "floppy" relative to the substrate; and the stability of the product complexes after bond cleavage is equal to what one estimates for Watson–Crick duplexes.

If the crystal structures mimic the ground state of the reaction, one might hope that the results of mutagenesis or functional group modification could be used to extrapolate from the observed structures to a proposal for the transition-state structure and catalytic mechanism. It has been shown that mutating any nucleotide except N_7 in the catalytic core of the hammerhead results in nearly complete loss (>10^3-fold reduction) of cleavage activity (Ruffner et al., 1990). Hence, mutagenesis between the naturally occurring nucleotides has proved to be too coarse in its effects to give useful structure–function relations, and an emphasis has been placed on function group alteration studies, in which modified nucleotides (Fig. 1) are incorporated at specific sites and their effect on ribozyme activity is determined.

Because substrate binding specificity is determined by Watson–Crick base pairing in stems I and III, it is assumed that modifications within the ribozyme core that reduce activity do so primarily by influencing catalysis, rather than substrate binding. Effects on activity could arise either from disruption of the overall tertiary structure or from destabilization of the transition state with minimal effect on the global structure. It is worth noting in this context that a 10^3-fold reduction in the rate of bond cleavage—which would slow the turnover rate from ~1 min^{-1} to ~1 day^{-1}—corresponds to a free energy change of ~4.1 kcal/mol at 25 °C, which is much smaller in magnitude than the transition-state activation energy of ~22 kcal/mol.

The most straightforward approach to extracting a coherent picture from available functional group modification data is to compare the relative values of kinetic constants between modified and unmodified ribozymes assayed under identical conditions. Comparison of absolute values of kinetic constants is more problematical, because they depend strongly on (1) the configuration of hammerhead used, i.e., which stems are closed by loops and which are left open; results have been reported on constructs with stem II closed, stem I closed, or stem III closed; (2) temperature and pH; increasing temperature from 25 °C to 37 °C or increasing pH from 7.5 to 8.0 will increase the rate of chemical cleavage approximately 4–5 fold (Hertel & Uhlenbeck, 1995); (3) divalent ion concentration; most kinetic constants have been measured at 10 mM [Mg^{2+}], whereas the K_m for the [Mg^{2+}]-dependence of the hammerhead activity is also ~10 mM, so that the activity is typically not maximized with respect to [Mg^{2+}]. Steady-state k_{cat} values have been reported commonly, in which case the turnover numbers incorporate substrate binding and product release as well as the rate of the chemical cleavage step. In some cases, measurements of the rate of chemical cleavage under single-turnover conditions are also reported. A summary of results is given in Table 1 and Figure 2. Some license of interpretation is implicit in the figures. In part, this is inevitable when one tries to extract a consensus picture from such a large body of data. In addition, it should be realized that not all functional group alterations are single-site in nature. For example, replacement of guanosine with inosine or replacement of adenosine with purine ribonucleotide deletes a single exocyclic amino group; such a change is likely to only result in the deletion of one, or at most two, hydrogen bonds, and the effects of the change can be ascribed to that particular amino group. On the other hand, replacement of guanosine with 2-aminopurine ribonucleotide removes O6 and also converts N1 from a hydrogen bond donor to an acceptor, thereby altering the donor–acceptor pattern of the base and making interpretation of the results more problematical. In such a case, both groups that are altered are highlighted in the figures, although it may be the case that only one of them is important for function. In addition to the single-site modifications cited in Table 1, the figures incorporate results in which replacement of several riboses simultaneously with 2′-deoxyriboses has little effect on activity (Perreault et al., 1990, 1991).

The break points of the color code for displaying reductions of activity, relative to wild-type, have been arbitrarily set to: red, less than 1% activity; yellow, 1–10% activity; magenta, 10–40%; and green, activity at least 40% of wild-type (i.e., little alteration). If these break points are converted to a value of $\Delta\Delta G$ at 25 °C using the relation $\Delta\Delta G = RT \ln(k_{mod}/k_{wt})$, they correspond to 0.5 kcal/mol for 40% activity, 1.4 kcal/mol for 10% activity, and 2.7 kcal/mol for 1% activity. As a point of reference, these can be compared to experimental values of free energy increments for hydrogen bond formation in RNA measured in a variety of contexts, which range from a few tenths of a kcal/mol (e.g., in a GCAA tetraloop) to ~1.5 kcal/mol in duplexes (for review, see Turner & Bevilacqua, 1993). If, hypothetically, a change in activity reflected an alteration in transition-state–

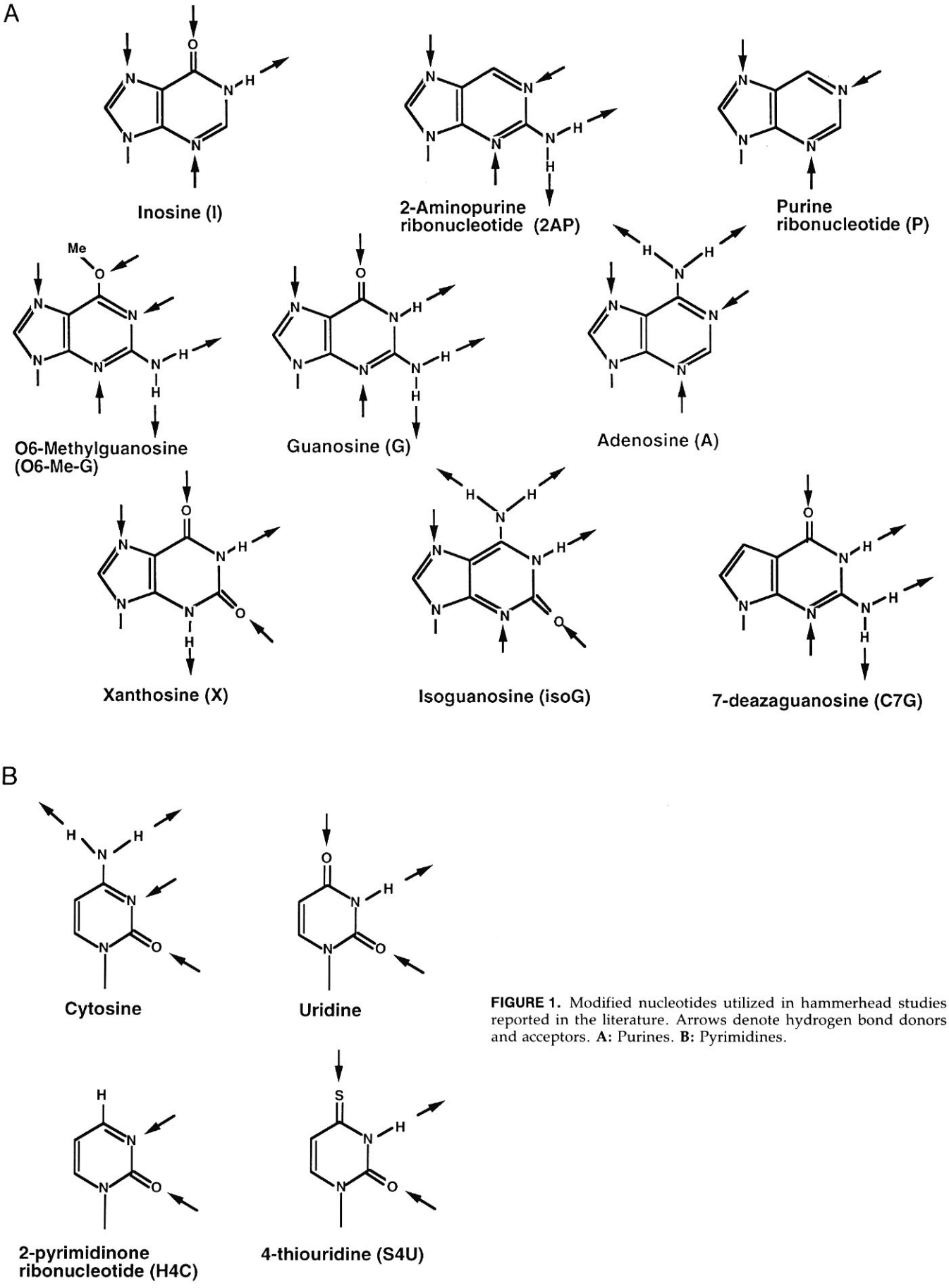

FIGURE 1. Modified nucleotides utilized in hammerhead studies reported in the literature. Arrows denote hydrogen bond donors and acceptors. **A:** Purines. **B:** Pyrimidines.

TABLE 1. Effects of modification of nucleotides in hammerhead core.

Nucleotide	Modification	Effect[a]	Relative activity[b]: k_{cat}(mod)/k_{cat}(WT)	K_m(mod)/K_m(WT)	k_{cat}(WT) (min^{-1})	K_m(WT) (μM)	Assay conditions: [TrisHCl] (mM)/[Mg^{2+}] (mM)	pH	T (°C)	Construct[c]	Reference
$N_{1.1}$	Rp-thioP	–	Qualitative loss of activity				50/10	8.0	37 or 50	III; 6,5,3	(Ruffner & Uhlenbeck, 1990)
	Rp-thioP	–	0.032		0.25	3.3	40/25 + 20 mM NaCl	7.5	37	none; 5,5,3	(Koizumi & Ohtsuka, 1991)
	Sp-thioP	o	3.5		0.25	3.3	40/25 + 20 mM NaCl	7.5	37	none; 5,5,3	(Koizumi & Ohtsuka, 1991)
	Rp-thioP	–	Loss of activity		2.8	0.023	50/20	7.4	30	II; 6,5,6	(Slim & Gait, 1991)
	Sp-thioP	o	Slight loss of activity		2.8	0.023	50/20	7.4	30	II; 6,5,6	(Slim & Gait, 1991)
C_3	H4C	(–)	0.14	0.6	0.29	0.8	50/10	7.9	37	III; 6,5,3	(Murray et al., 1995)
U_4	2′-deoxy	o	0.52 (k_{cat}/K_m)		2.9 (k_{cat}/K_m)		40/10	7.5	25	II; 5,4,5	(Tanaka et al., 1993)
	H4C	(–)	0.16	3.1	0.29	0.8	50/10	7.9	37	III; 6,5,3	(Murray et al., 1995)
	S4U	(–)	0.33	0.9	0.29	0.8	50/10	7.9	37	III; 6,5,3	(Murray et al., 1995)
G_5	2′-deoxy	– –	0.0026	0.26	2.3	0.15	50/10	7.5	25	II; 5,4,5	(Williams et al., 1992)
	2′-deoxy	– –	0.0037		2.8	0.078	50/10	7.5	25	II; 5,4,5	(Tuschl et al., 1993)
	2′-deoxy	–	0.07	1.1	2.6	0.92	75/10	7.5	37	II; 6,4,7	(Perreault et al., 1991)
	2′-deoxy	– –	0.0061ss/0.015st	7.8	0.88ss/0.11st	0.88	50/10	8.0	55ss/37st	III; 6,5,3	(Fu & McLaughlin, 1992b)
	2′-deoxy	–	0.024 (k_{cat}/K_m)		2.9 (k_{cat}/K_m)		40/10	7.5	25	II; 5,4,5	(Tanaka et al., 1993)
	ara-G	– –	<0.0001		1.5	0.051	50/10	7.5	25	II; 5,4,5	(Fu et al., 1994)
	2′-F	–	0.017	2.0	2.3		50/10	7.5	25	II; 5,4,5	(Williams et al., 1992)
	2′-amino	(–)	0.35	1.0	2.3		50/10	7.5	25	II; 5,4,5	(Williams et al., 1992)
	I	– –	0.004	5.8	2.8	0.078	50/10	7.5	25	II; 5,4,5	(Tuschl et al., 1993)
	I	–	0.015ss/0.039st	4.8	0.88ss/0.11st	0.078	50/10	8.0	55ss/37st	III; 6,5,3	(Fu & McLaughlin, 1992b)
	I	–	0.020 (k_{cat}/K_m)		2.9 (k_{cat}/K_m)		40/10	7.5	25	II; 5,4,5	(Tanaka et al., 1993)
	2′-deoxy-I	– –	0.003 (k_{cat}/K_m)		2.9 (k_{cat}/K_m)		40/10	7.5	25	II; 5,4,5	(Tanaka et al., 1993)
	2AP	– –	0.0018	5.0	2.8	0.078	50/10	7.5	25	II; 5,4,5	(Tuschl et al., 1993)
	X	– –	0.0014		2.8	0.078	50/10	7.5	25	II; 5,4,5	(Tuschl et al., 1993)
	isoG	– –	<0.001		2.8	0.078	50/10	7.5	25	II; 5,4,5	(Tuschl et al., 1993)
	C7G	(–)	0.28ss/0.34st	2.2	0.69ss/0.079st	1.4	50/10	8.0	55ss/37st	III; 6,5,3	(Fu et al., 1993)
	O6-Me-G	–	0.014	1.8	1.5	0.074	50/10	7.5	25	II; 5,4,5	(Grasby et al., 1993)
A_6	2′-deoxy	(–)	nd/0.39st		0.88ss/0.11st		50/10	8.0	55ss/37st	III; 6,5,3	(Fu & McLaughlin, 1992b)
	isoG	(–)	0.44		2.5		50/10	7.5	25	II; 5,4,5	(Ng et al., 1994)
	2AP	–	0.03		2.5		50/10	7.5	25	II; 5,4,5	(Ng et al., 1994)
	G	–	0.013		2.5		50/10	7.5	25	II; 5,4,5	(Ng et al., 1994)
	C7A	–	0.010ss/0.028st		0.78ss/0.053st		50/10	8.0	55ss/37st	III; 6,5,3	(Fu & McLaughlin, 1992a)
	Purine	o	nd/0.79		0.88ss/0.11st		50/10	8.0	55ss/37st	III; 6,5,3	(Fu & McLaughlin, 1992b)
U_7	dU	o	3.3 (k_{cat}/K_m)		2.9 (k_{cat}/K_m)		40/10	7.5	25	II; 5,4,5	(Tanaka et al., 1993)
	H4C	o	0.69	0.9	0.29	0.8	50/10	7.9	37	III; 6,5,3	(Murray et al., 1995)
G_8	2′-deoxy	– –	0.002	0.33	2.3	0.15	50/10	7.5	25	II; 5,4,5	(Williams et al., 1992)
	2′-deoxy	– –	0.008		3.0		50/10	7.5	25	II; 5,4,5	(Tuschl et al., 1993)
	2′-deoxy	o	0.46	1.0	2.6	0.92	75/10	7.5	37	II; 6,4,7	(Perreault et al., 1991)
	2′-deoxy	–	0.051ss/0.046st	15	0.88ss/0.11st	0.88	50/10	8.0	55ss/37st	III; 6,5,3	(Fu & McLaughlin, 1992b)
	2′-deoxy	–	0.069 (k_{cat}/K_m)		2.9 (k_{cat}/K_m)		40/10	7.5	25	II; 5,4,5	(Tanaka et al., 1993)
	2′-F	– –	0.003	1.47	2.3		50/10	7.5	25	II; 5,4,5	(Williams et al., 1992)
	2′-amino	–	0.069	1.23	2.3		50/10	7.5	25	II; 5,4,5	(Williams et al., 1992)
	ara-G	–	0.09	0.22	1.5	0.051	50/10	7.5	25	II; 5,4,5	(Fu et al., 1994)
	I	(–)	0.37	3.2	2.8	0.078	50/10	7.5	25	II; 5,4,5	(Tuschl et al., 1993)
	I	–	~0.08				50/10	7.4	30	I; 6,5,6	(Slim & Gait, 1992)
	I	(–)	nd/0.36st		0.88ss/0.11st		50/10	8.0	55ss/37st	III; 6,5,3	(Fu & McLaughlin, 1992b)
	I	o	0.79 (k_{cat}/K_m)		2.9 (k_{cat}/K_m)		40/10	7.5	25	II; 5,4,5	(Tanaka et al., 1993)
	2′-deoxy-I	–	0.028 (k_{cat}/K_m)		2.9 (k_{cat}/K_m)		40/10	7.5	25	II; 5,4,5	(Tanaka et al., 1993)
	2AP	– –	0.0061	0.8	2.8	0.078	50/10	7.5	25	II; 5,4,5	(Tuschl et al., 1993)
	X	– –	0.0024		2.8	0.078	50/10	7.5	25	II; 5,4,5	(Tuschl et al., 1993)
	isoG	– –	0.0017		2.8	0.078	50/10	7.5	25	II; 5,4,5	(Tuschl et al., 1993)
	C7G	(–)	0.14ss/0.39st	0.78	0.69ss/0.079st	1.4	50/10	8.0	55ss/37st	III; 6,5,3	(Fu et al., 1993)
	O6-Me-G	–	0.013	1.5	1.5	0.074	50/10	7.5	25	II; 5,4,5	(Grasby et al., 1993)
A_9	2′-deoxy	–	0.05	1.0	2.6	0.92	75/10	7.5	37	II; 6,4,7	(Perreault et al., 1991)
	2′-deoxy	o	nd/1.91st		0.88ss/0.11st		50/10	8.0	55ss/37st	III; 6,5,3	(Fu & McLaughlin, 1992b)
	2′-deoxy	o	0.51	1.2	41	0.22	50/10(Mn^{2+})	7.5	25	II; 5,4,5	(Olsen et al., 1991)
	2′-F	o	0.93	1.2	41	0.22	50/10(Mn^{2+})	7.5	25	II; 5,4,5	(Olsen et al., 1991)

	isoG	o	0.58		2.5		50/10	7.5	25	II; 5,4,5	(Ng et al., 1994)
	2AP	(−)	0.20				50/10	7.5	25	II; 5,4,5	(Ng et al., 1994)
	G	−−	0.0085				50/10	7.5	25	II; 5,4,5	(Ng et al., 1994)
	C7A	(−)/o	0.10ss/0.57st		0.78ss/0.053st		50/10	8.0	55ss/37st	III; 6,5,3	(Fu & McLaughlin, 1992a)
	Purine	o	nd/0.90st		0.88ss/0.11st		50/10	8.0	55ss/37st	III; 6,5,3	(Fu & McLaughlin, 1992b)
	Purine	(−)	~0.17				50/10	7.4	30	I; 6,5,6	(Slim & Gait, 1992)
	Rp-thioP		Qualitative loss of activity				50/10	8.0	37 or 50	III; 6,5,3	(Ruffner & Uhlenbeck, 1990)
$N_{10.1}$	C-G for G-C bp	−−	0.004	0.92	3.1	0.087	50/10	7.5	25	II; 5,4,5	(Tuschl & Eckstein, 1993)
	C-G for G-C bp	−	0.055				50/10	8.0	37	III; 6,5,3	(Ruffner et al., 1990)
G_{12}	2′-deoxy	o	1.4 (k_{cat}/K_m)		2.9 (k_{cat}/K_m)		40/10	7.5	25	II; 5,4,5	(Tanaka et al., 1993)
	2′-deoxy	o	1.5		2.8	0.078	50/10	7.5	25	II; 5,4,5	(Tuschl et al., 1993)
	ara-G	(−)	0.15	0.78	1.5	0.051	50/10	7.5	25	II; 5,4,5	(Fu et al., 1994)
	I	−−	0.003	4.1	2.8	0.078	50/10	7.5	25	II; 5,4,5	(Tuschl et al., 1993)
	I	−	≤0.03				50/10	7.4	30	I; 6,5,6	(Slim & Gait, 1992)
	I	−	0.017ss/0.020st	7.8	0.69ss/0.079st	1.4	50/10	8.0	55ss/37st	III; 6,5,3	(Fu et al., 1993)
	I	−	0.024 (k_{cat}/K_m)		2.9 (k_{cat}/K_m)		40/10	7.5	25	II; 5,4,5	(Tanaka et al., 1993)
	2′-deoxy-I	(−)	0.13 (k_{cat}/K_m)		2.9 (k_{cat}/K_m)		40/10	7.5	25	II; 5,4,5	(Tanaka et al., 1993)
	2AP	−−	0.0004		2.8	0.078	50/10	7.5	25	II; 5,4,5	(Tuschl et al., 1993)
	X	−−	0.0017		2.8	0.078	50/10	7.5	25	II; 5,4,5	(Tuschl et al., 1993)
	isoG	−−	0.0004		2.8	0.078	50/10	7.5	25	II; 5,4,5	(Tuschl et al., 1993)
	C7G	o	0.68ss/0.77st	0.86	0.69ss/0.079st	1.4	50/10	8.0	55ss/37st	III; 6,5,3	(Fu et al., 1993)
	O6-Me-G	−	0.017	1.1	1.5	0.074	50/10	7.5	25	II; 5,4,5	(Grasby et al., 1993)
A_{13}	2′-deoxy	(−)	0.34	0.76	41	0.22	50/10(Mn^{2+})	7.5	25	II; 5,4,5	(Olsen et al., 1991)
	2′-F	o	0.54	1.1	41	0.22	50/10(Mn^{2+})	7.5	25	II; 5,4,5	(Olsen et al., 1991)
	isoG	o	0.61		2.5		50/10	7.5	25	II; 5,4,5	(Ng et al., 1994)
	2AP	(−)	0.15				50/10	7.5	25	II; 5,4,5	(Ng et al., 1994)
	G	−−	<0.0008				50/10	7.5	25	II; 5,4,5	(Ng et al., 1994)
	C7A	(−)/o	0.24ss/0.49st		0.78ss/0.053st		50/10	8.0	55ss/37st	III; 6,5,3	(Fu & McLaughlin, 1992a)
	Purine	−/(−)	0.056ss/0.14st	1.4	0.69ss/0.079st	1.4	50/10	8.0	55ss/37st	III; 6,5,3	(Fu et al., 1993)
	Purine	−	~0.08				50/20	7.4	30	I; 6,5,6	(Slim & Gait, 1992)
	Rp-thioP	−	Qualitative loss of activity				50/10	8.0	37 or 50	III; 6,5,3	(Ruffner & Uhlenbeck, 1990)
A_{14}	2′-deoxy	(−)	0.49	0.81	41	0.22	50/10(Mn^{2+})	7.5	25	II; 5,4,5	(Olsen et al., 1991)
	2′-F	(−)	0.34	0.85	41	0.22	50/10(Mn^{2+})	7.5	25	II; 5,4,5	(Olsen et al., 1991)
	isoG	(−)	0.43		2.5		50/10	7.5	25	II; 5,4,5	(Ng et al., 1994)
	C7A	(−)	0.07ss/0.31st		0.78ss/0.053st		50/10	8.0	55ss/37st	III; 6,5,3	(Fu & McLaughlin, 1992a)
	Purine	−/(−)	0.081ss/0.30st	1.2	0.69ss/0.079st	1.4	50/10	8.0	55ss/37st	III; 6,5,3	(Fu et al., 1993)
	Purine	o	~1.2				50/20	7.4	30	I; 6,5,6	(Slim & Gait, 1992)
	Rp-thioP	−	Qualitative loss of activity				50/10	8.0	37 or 50	III; 6,5,3	(Ruffner & Uhlenbeck, 1990)
$A_{15.1}$	2′-deoxy	(−)	0.12		41	0.19	50/10(Mn^{2+})	7.5	25	II; 5,4,5	(Olsen et al., 1991)
	2′-F	(−)	0.10		41	0.19	50/10(Mn^{2+})	7.5	25	II; 5,4,5	(Olsen et al., 1991)
	C7A	(−)	0.12ss/0.31st		0.78ss/0.053st		50/10	8.0	55ss/37st	III; 6,5,3	(Fu & McLaughlin, 1992a)
	Purine	−/(−)	0.022ss/0.16st	1.3	0.69ss/0.079st	1.4	50/10	8.0	55ss/37st	III; 6,5,3	(Fu et al., 1993)
	Purine	−	≤0.03				50/20	7.4	30	I; 6,5,6	(Slim & Gait, 1992)
$U_{16.1}$	T(2′-deoxy)	−	0.046	8.0	2.6	0.92	75/10	7.5	37	II; 6,4,7	(Perreault et al., 1991)
	H4C	−−	None detected		0.29	0.8	50/10	7.9	37	III; 6,5,3	(Murray et al., 1995)
	S4U	o	0.66	1.2	0.29	0.8	50/10	7.9	37	III; 6,5,3	(Murray et al., 1995)
C_{17}	I	(−)	0.17				40/25 + 20 mM NaCl	7.5	37	none; 5,5,3	(Koizumi & Ohtsuka, 1991)
	G	−	0.040				40/25 + 20 mM NaCl	7.5	37	none; 5,5,3	(Koizumi & Ohtsuka, 1991)
	G	−−	<0.003				50/10	8.0	37	III; 6,5,3	(Ruffner et al., 1990)
	A	o	0.7				50/10	8.0	37	III; 6,5,3	(Ruffner et al., 1990)
	U	−	0.05				50/10	8.0	37	III; 6,5,3	(Ruffner et al., 1990)
	H4C	(−)	0.29	2.9	0.29	0.8	50/10	7.9	37	III; 6,5,3	(Murray et al., 1995)

[a] Range of effect, given as fractional loss of activity: −−, <0.01; −, 0.1—0.01; (−), 0.4-0.1; o, >0.4, as in Figure 2A.

[b] Relative activity is k_{cat} of modified ribozyme divided by k_{cat} of wild type, unless noted otherwise. Values are from steady-state measurements unless noted otherwise. ss, steady state, st, single-turnover or stoichiometric assay conditions. Units of k_{cat}/K_m are not given in Tanaka et al. (1993).

[c] Construct is summarized: numerical designation of stem(s) that is(are) closed with a loop; predicted number of Watson—Crick base pairs in stems I, II, III, respectively. Abbreviations for modifications are as in Figure 1.

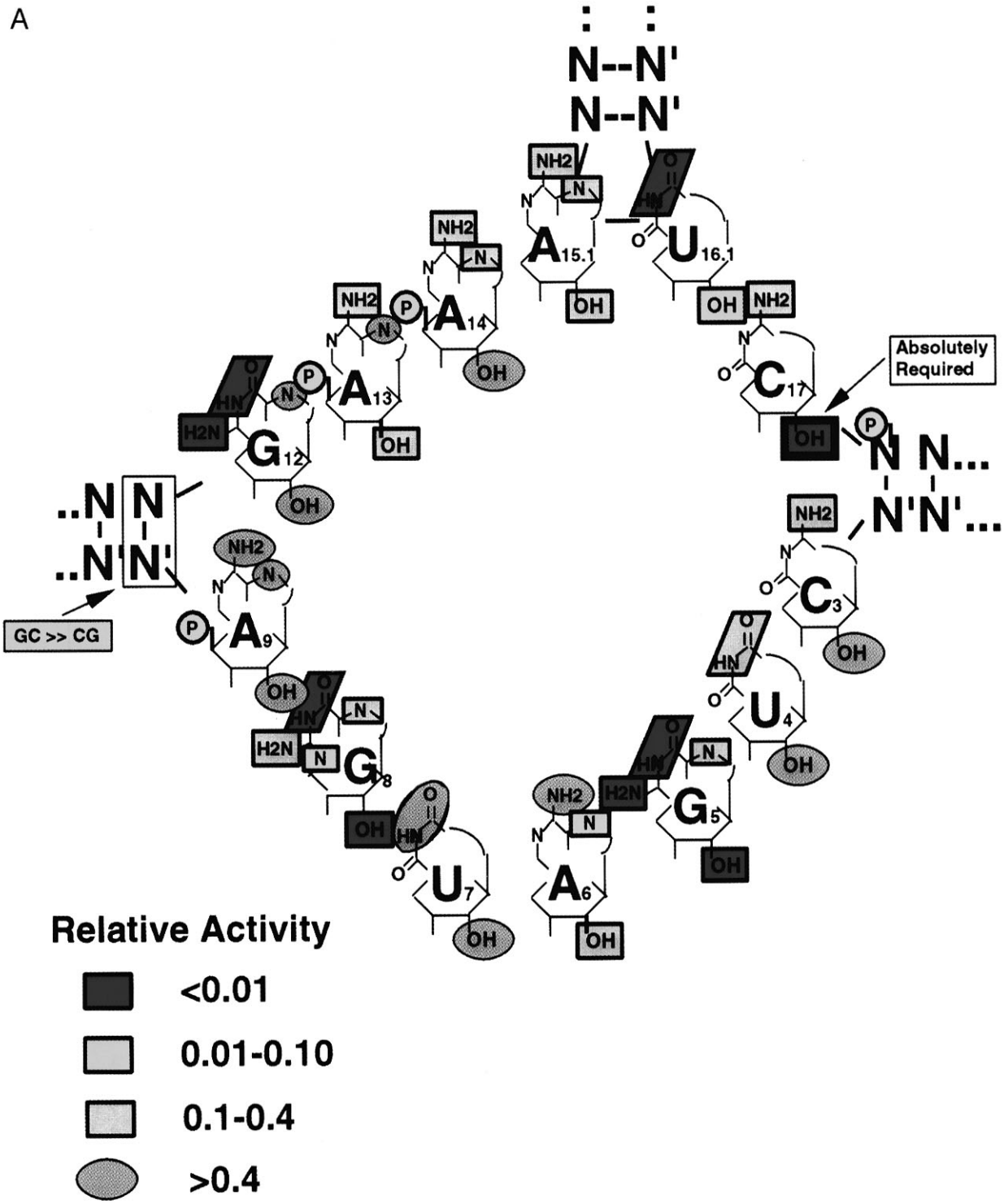

FIGURE 2. Effects of functional group modification on hammerhead activity, as summarized in Table 1. Color coding of relative activity resulting from modification of functional group is: red, <0.01 × wild-type activity; yellow, 0.01–0.1 × wild-type activity; magenta, 0.1–0.4 × wild-type activity; green, >0.4 × wild-type activity. In some cases, several reports of the same modification give different values for the effect on k_{cat}; an effort has been made to illustrate the majority result, but the reader is encouraged to refer to the original literature in such cases. N3 of G_8 has been colored yellow to emphasize the large difference between the activities of ribozymes with xanthosine versus those with inosine at this position, although the effect is not strictly assignable solely to the change from hydrogen bond acceptor to donor at N3; note that the difference between effects of xanthosine and inosine are not as large at other guanosine positions. Phosphates whose replacement with thiophosphates results in a qualitative loss of activity have been colored yellow. **A:** Consensus sequence of hammerhead core. **B:** Stereo view of domain 1 of the hammerhead, in approximately same view as in Pley et al. (1994). RNA nucleotides $C_{2.1}$-U_7 are blue; DNA nucleotides $G_{16.2}$-$G_{1.1}$ are cyan. Probable hydrogen bonds are shown in orange. Scissile phosphorus–oxygen bond is shown in gold. **C:** Stereo view of domain 2. Nucleotides U_7-$G_{10.1}$ are colored cyan; nucleotides $C_{11.1}$-A_{14} are colored blue; divalent ion is colored gold.

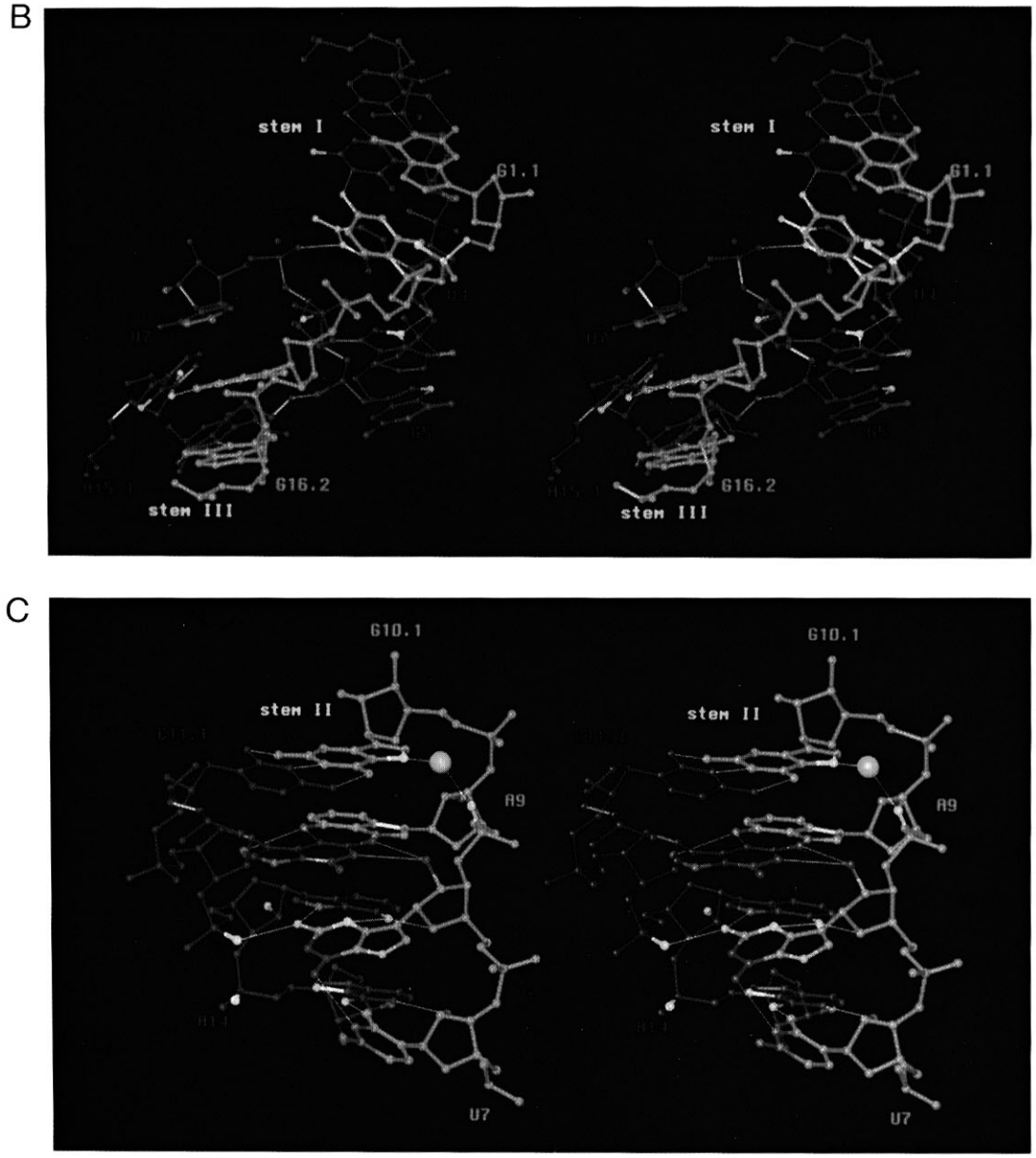

FIGURE 2. (*continued.*)

ground-state energy difference, and the change was due solely to deletion of specific hydrogen bonds that were present in the transition state but absent in the ground state, then a reduction to 40% activity might be a consequence of loss of a single weak hydrogen bond, reduction to 10% activity from loss of a single strong hydrogen bond, and so forth.

If it were the case that those groups whose alteration results in substantial decrease in activity were involved in specific tertiary interactions, their interpretation could (arguably) be straightforward: the modification may affect activity by disrupting the ribozyme structure. Such apparent correlation is suggested in the region around a divalent ion binding site of domain 2, where, in the crystal structures, N7 of $G_{10.1}$ and the *pro*-Rp phosphate oxygen of A_9 coordinate the divalent ion, and the nearby 2′ hydroxyl of G_8 mediates specific tertiary interactions (Fig. 2C). The ability of modifications that are remote from the cleavage site to substantially reduce catalytic activity also suggests that cooperative, "action at a distance" effects are an intrinsic feature of hammerhead catalysis. However, many of the effects of other modifications in domain 2 do not submit to an equally straightforward rationalization. And, nearer the cleavage site in domain 1, the hammerhead structures exhibit even greater discord with the functional group modification data. Of particular note is G_5; any alteration of its exocyclic groups results in $\sim 10^3$-fold reduction of activity; yet, these groups interface only to solvent in the crystal struc-

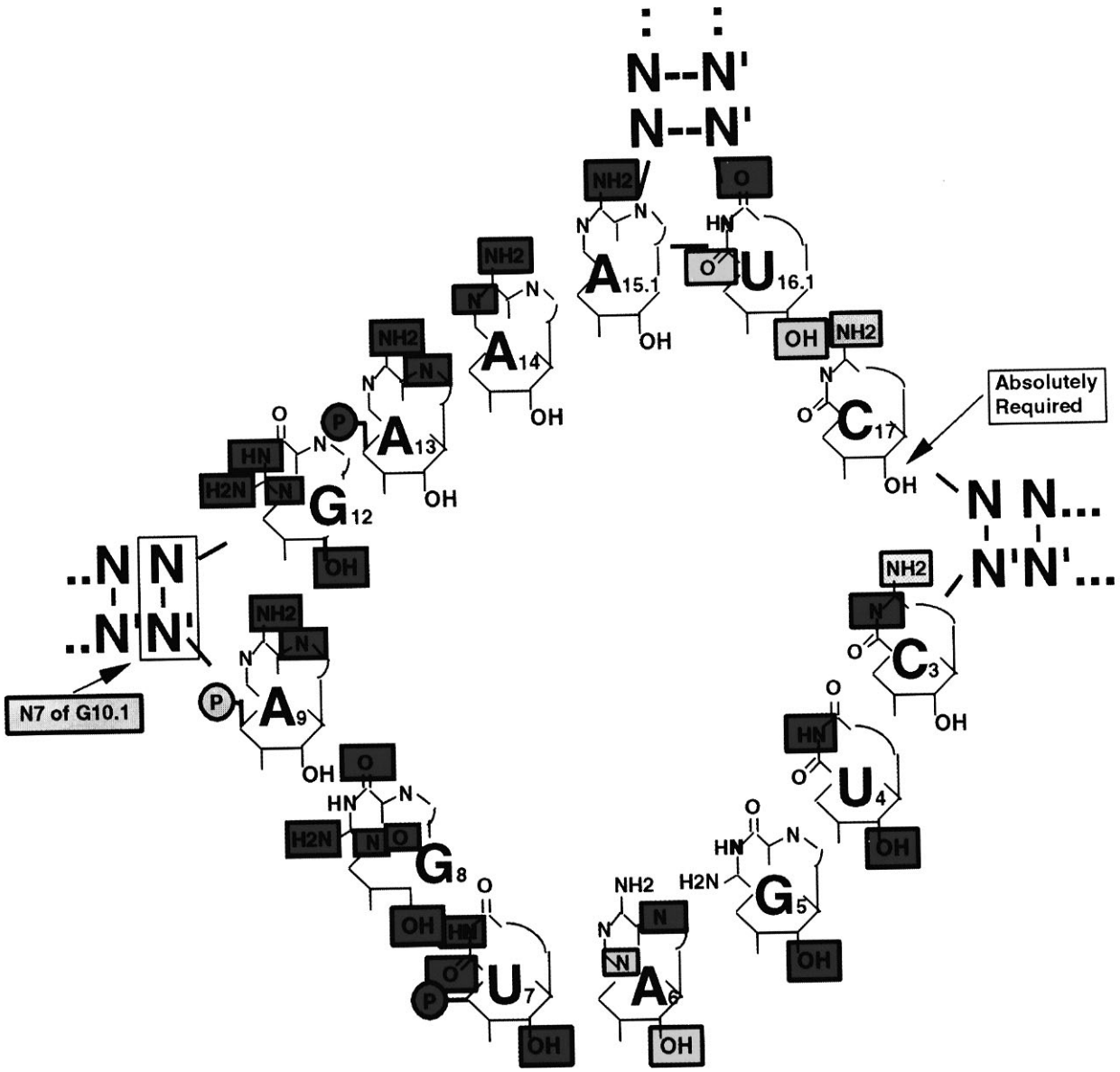

FIGURE 3. Functional groups that are involved in specific interactions in the hammerhead ribozyme structures. Red, groups that are involved in apparent hydrogen bonds in both the RNA–DNA structure (Pley et al., 1994) and the all-RNA structure (Scott et al., 1995); yellow, groups that form hydrogen bonds in the all-RNA structure only, due to the 2′-OH of $U_{16.1}$; magenta, groups that are identified as divalent cation ligands in both structures; cyan, group that is identified as a ligand for a potential hydrated Mg^{2+} ion in all-RNA structure only; purple, group that forms hydrogen bond in both structures and is also a ligand for a potential hydrated Mg^{2+} ion in all-RNA structures. Note that, due to the different nature of the information displayed, interpretation of the color coding differs from that of Figure 2A.

tures, raising the specter that a significant conformational shift may be required to allow the guanosine base to interact with other parts of the ribozyme and influence catalysis (Fig. 2B). Hence, in many cases, functional groups that are identified as important for catalysis are not involved in specific (ground state) tertiary interactions (Fig. 3); nor are they clustered together in a topography suggesting an obvious "lock and key" complementarity to the anticipated transition state of the cleavage reaction.

Consequently, although the overall tertiary structure of the hammerhead ribozyme is now known, the mechanism by which such a simple structure catalyzes a specific bond cleavage still remains a mystery. There is no cogent argument that suggests either the crystallographic or the enzymatic data are incorrect or irrelevant; hence, it is incumbent that proposals of catalytic mechanism should satisfactorily rationalize both sets of data. The inability of currently available data to unambiguously reveal the mechanism of catalysis, al-

though initially disappointing, may serve to focus future endeavors in this area. On the enzymatic side, one can hope that further characterizing ribozymes with modifications that dramatically alter activity, in the context of both the detailed kinetic scheme (Hertel et al., 1994) and probes of global conformation (Tuschl et al., 1994; Bassi et al., 1995) of the molecule, will help clarify the role of functional groups of the ribozyme core. On the structural side, one can hope that efforts to solve structures with transition-state or reaction intermediate analogues of the hammerhead ribozyme, thereby giving structural information that more closely mimics a catalytic conformation, will prove successful eventually.

ACKNOWLEDGMENTS

This work was supported by NIH grant AI-30606. I thank Kevin Flaherty for assistance in making figures and Dr. Dan Herschlag for constructive discussions.

REFERENCES

Amiri KM, Hagerman PJ. 1994. Global conformation of a self-cleaving hammerhead RNA. *Biochemistry 33*:13172–13177.

Bassi GS, Mollegaard NE, Murchie AI, von KE, Lilley DM. 1995. Ionic interactions and the global conformations of the hammerhead ribozyme. *Nature Struct Biol 2*:45–55.

Buzayan JM, Hampel A, Bruening G. 1986. Nucleotide sequence and newly formed phosphodiester bond of spontaneously ligated satellite tobacco ringspot virus RNA. *Nucleic Acids Res 14*:9729–9743.

Dahm SC, Derrick WB, Uhlenbeck OC. 1993. Evidence for the role of solvated metal hydroxide in the hammerhead cleavage mechanism. *Biochemistry 32*:13040–13045.

Dahm SC, Uhlenbeck OC. 1990. Characterization of deoxy- and ribo-containing oligonucleotide substrates in the hammerhead self-cleavage reaction. *Biochimie 72*:819–823.

Dahm SC, Uhlenbeck OC. 1991. Role of divalent metal ions in the hammerhead RNA cleavage reaction. *Biochemistry 30*:9464–9469.

Fu DJ, McLaughlin LW. 1992a. Importance of specific adenosine N7-nitrogens for efficient cleavage by a hammerhead ribozyme. A model for magnesium binding. *Biochemistry 31*:10941–10949.

Fu DJ, McLaughlin LW. 1992b. Importance of specific purine amino and hydroxyl groups for efficient cleavage by a hammerhead ribozyme. *Proc Natl Acad Sci USA 89*:3985–3989.

Fu DJ, Rajur SB, McLaughlin LW. 1993. Importance of specific guanosine N7-nitrogens and purine amino groups for efficient cleavage by a hammerhead ribozyme. *Biochemistry 32*:10629–10637.

Fu DJ, Rajur SB, McLaughlin LW. 1994. Activity of the hammerhead ribozyme upon inversion of the stereocenters for the guanosine 2′-hydroxyls. *Biochemistry 33*:13903–13909.

Gautheret D, Konings D, Gutell RR. 1994. A major family of motifs involving G·A mismatches in ribosomal RNA. *J Mol Biol 242*:1–8.

Grasby JA, Jonathan P, Butler G, Gait MJ. 1993. The synthesis of oligoribonucleotides containing O6-methylguanosine: The role of conserved guanosine residues in hammerhead ribozyme cleavage. *Nucleic Acids Res 21*:4444–4450.

Hertel KJ, Herschlag D, Uhlenbeck OC. 1994. A kinetic and thermodynamic framework for the hammerhead ribozyme reaction. *Biochemistry 33*:3374–3385.

Hertel KJ, Uhlenbeck OC. 1995. The internal equilibrium of the hammerhead ribozyme reaction. *Biochemistry 34*:1744–1749.

Hutchins CJ, Rathjen PD, Forster AC, Symons RH. 1986. Self-cleavage of plus and minus RNA transcripts of avocado sunblotch viroid. *Nucleic Acids Res 14*:3627–3640.

Koizumi M, Ohtsuka E. 1991. Effects of phosphorothioate and 2-amino groups in hammerhead ribozymes on cleavage rates and Mg^{2+} binding. *Biochemistry 30*:5145–5150.

Murray JB, Adams CJ, Arnold JRP, Stockley PG. 1995. The roles of the conserved pyrimidine bases in hammerhead ribozyme catalysis: Evidence for a magnesium ion-binding site. *Biochem J 311*:487–494.

Ng MM, Benseler F, Tuschl T, Eckstein F. 1994. Isoguanosine substitution of conserved adenosines in the hammerhead ribozyme. *Biochemistry 33*:12119–12126.

Olsen DB, Benseler F, Aurup H, Pieken WA, Eckstein F. 1991. Study of a hammerhead ribozyme containing 2′-modified adenosine residues. *Biochemistry 30*:9735–9741.

Perreault JP, Labuda D, Usman N, Yang JH, Cedergren R. 1991. Relationship between 2′-hydroxyls and magnesium binding in the hammerhead RNA domain: A model for ribozyme catalysis. *Biochemistry 30*:4020–4025.

Perreault JP, Wu TF, Cousineau B, Ogilvie KK, Cedergren R. 1990. Mixed deoxyribo- and ribo-oligonucleotides with catalytic activity. *Nature 344*:565–567.

Pley HW, Flaherty KM, McKay DB. 1994. Three-dimensional structure of a hammerhead ribozyme. *Nature 372*:68–74.

Quigley GJ, Rich A. 1976. Structural domains of transfer RNA molecules. *Science 194*:796–806.

Ruffner DE, Stormo GD, Uhlenbeck OC. 1990. Sequence requirements of the hammerhead RNA self-cleavage reaction. *Biochemistry 29*:10695–10702.

Ruffner DE, Uhlenbeck OC. 1990. Thiophosphate interference experiments locate phosphates important for the hammerhead RNA self-cleavage reaction. *Nucleic Acids Res 18*:6025–6029.

Scott WG, Finch JT, Klug A. 1995. The crystal structure of an all-RNA hammerhead ribozyme: A proposed mechanism for RNA catalytic cleavage. *Cell 81*:991–1002.

Slim G, Gait MJ. 1991. Configurationally defined phosphorothioate-containing oligoribonucleotides in the study of the mechanism of cleavage of hammerhead ribozymes. *Nucleic Acids Res 19*:1183–1188.

Slim G, Gait MJ. 1992. The role of the exocyclic amino groups of conserved purines in hammerhead ribozyme cleavage. *Biochem Biophys Res Commun 183*:605–609.

Tanaka H, Hosaka H, Takahashi R, Imamura Y, Takai K, Yokoyama S, Takaku H. 1993. Importance of specific purine–pyrimidine amino and hydroxyl groups for efficient cleavage by a hammerhead ribozyme. *Nucleic Acids Symp Ser 1993*:175–176.

Turner DH, Bevilacqua PC. 1993. Thermodynamic considerations for evolution by RNA. In: Gesteland RF, Atkins JF, eds. *The RNA world*. Cold Spring Harbor, New York: Cold Spring Harbor Laboratory Press. pp 447–464.

Tuschl T, Eckstein F. 1993. Hammerhead ribozymes: Importance of stem-loop II for activity. *Proc Natl Acad Sci USA 90*:6991–6994.

Tuschl T, Gohlke C, Jovin TM, Westhof E, Eckstein F. 1994. A three-dimensional model for the hammerhead ribozyme based on fluorescence measurements. *Science 266*:785–789.

Tuschl T, Ng MM, Pieken W, Benseler F, Eckstein F. 1993. Importance of exocyclic base functional groups of central core guanosines for hammerhead ribozyme activity. *Biochemistry 32*:11658–11668.

van Tol H, Buzayan JM, Feldstein PA, Eckstein F, Bruening G. 1990. Two autolytic processing reactions of a satellite RNA proceed with inversion of configuration. *Nucleic Acids Res 18*:1971–1975.

Williams DM, Pieken WA, Eckstein F. 1992. Function of specific 2′-hydroxyl groups of guanosines in a hammerhead ribozyme probed by 2′ modifications. *Proc Natl Acad Sci USA 89*:918–921.