A Nucleophile Activation Dyad in Ribonucleases

A COMBINED X-RAY CRYSTALLOGRAPHIC/ab Initio QUANTUM CHEMICAL STUDY*

Received for publication, June 28, 2002
Published, JBC Papers in Press, July 16, 2002, DOI 10.1074/jbc.M206461200

Pierre Mignon‡, Jan Steyaert§, Remy Loris§, Paul Geerlings‡¶, and Stefan Loverix‡¶

From the ‡Eenheid Algemene Chemie (ALGC), Faculteit Wetenschappen, Vrije Universiteit Brussel, Pleinlaan 2, 1050 Brussels, Belgium and §Dienst Ultrastructuur, VIB (Vlaams Interuniversitair Instituut Biotechnologie), Vrije Universiteit Brussel, Paardenstraat 65, B-1640 Sint-Genesius-Rode, Belgium

Ribonucleases (RNases) catalyze the cleavage of the phosphodiester bond in RNA up to $10^{15}$-fold, as compared with the uncatalyzed reaction. High resolution crystal structures of these enzymes in complex with 3'-mononucleotide substrates demonstrate the accommodation of the nucleophilic 2'-OH group in a binding pocket comprising the catalytic base (glutamate or histidine) and a charged hydrogen bond donor (lysine or histidine). Ab initio quantum chemical calculations performed on such Michaelis complexes of the mammalian RNase A (EC 3.1.27.5) and the microbial RNase T$_1$ (EC 3.1.27.3) show negative charge build up on the 2'-oxygen upon substrate binding. The increased nucleophilicity results from stronger hydrogen bonding to the catalytic base, which is mediated by a hydrogen bond from the charged donor. This hitherto unrecognized catalytic dyad in ribonucleases constitutes a general mechanism for nucleophile activation in both enzymic and RNA-catalyzed phosphoryl transfer reactions.

RNases have been the subject of landmark research in areas ranging from basic protein chemistry to enzymeology to protein folding and crystallography. These enzymes catalyze the intramolecular nucleophilic displacement of the 5’-leaving group by the attacking 2’-hydroxyl group in RNA, forming a 2’,3’-cyclophosphate (see Fig. 1). The nucleophilic attack occurs in line, in a postulated trigonal bipyramidal transition state, implying a catalytic base and acid on either side of the scissile bond (1, 2). X-ray crystallographical and site-directed mutagenesis experiments have provided substantial insight in the structure-function relationship of two unrelated families of ribonucleases. RNase A, (bovine pancreatic ribonuclease A) (EC 3.1.27.5) (3) and RNase T$_1$, (EC 3.1.27.3) (4, 5) (and references therein) are the best characterized members of the mammalian and the microbial enzymes, respectively. The mammalian enzymes depend on two histidines for acid/base catalysis, whereas a histidine/glutamic acid pair is found in the microbial enzymes (6).

The active site of RNase T$_1$ is composed of the side chains of Tyr-38, His-40, Glu-58, Arg-77, His-92, and Phe-100 (see Fig. 2n). With the exception of Arg-77, all these amino acids have been shown to take part in catalysis (5). Both the His-40 and Glu-58 side chains are in the direct vicinity of the 2’-nucleophile. pH dependence studies have shown Glu-58 to be unprotonated and His-40 to be protonated at the onset of catalysis, proving that Glu-58 is the catalytic base accepting a proton from the 2’-nucleophile (7). The protonated His-92 located at the opposite side of the active site functions as the catalytic acid. Removal of the His-40 side chain leads to a 6500-fold decrease in the second-order rate constant $k_{\text{cat}}/K_m$ suggesting an important catalytic role for this residue in the wild type enzyme. His-40 is believed to polarize the 2’-hydroxyl group and to properly orient Glu-58 toward the substrate (8, 9). However, the actual role of this residue is not totally clarified at present.

Despite the lack of sequential homology between RNase A and RNase T$_1$, their active site architectures show considerable similarities (Fig. 2). In RNase A, the protonated His-119 and the unprotonated His-12 form the catalytic acid/base pair, respectively. His-12 superimposes with its RNase T$_1$ counterpart Glu-58 (Fig. 2c). Lys-41, another important residue in RNase A, has been shown to donate a single hydrogen bond to the rate-limiting transition state of the reaction (10). The lack of large thioeffects for the RNase A-catalyzed reaction (11) argues against important catalytic interactions with the nonbridging oxygens. Moreover, crystallographic studies of RNase A complexed with a 3’-mononucleotide substrate (3’-CMP (12) and 3’-UMP (13)) or with the transition state analog uridine vanadate (14) persistently show a short hydrogen bond of about 2.7 Å between N$_\text{7}$ of Lys-41 and the 2’-oxygen, much like His-40 in RNase T$_1$ (Fig. 2c). Early molecular dynamics simulations on RNase A complexed with the dinucleotide substrate CaP support these observations (15). However, Lys-41 is still considered to stabilize negative charge build up on one of the nonbridging phosphoryl oxygens (3), and its precise role in catalysis remains controversial.

Because RNase-catalyzed phosphodiester cleavage is not a dissociative process (16), the nucleophilic attack contributes largely to the rate-limiting step of the reaction. The nucleophilic attack on hard centers such as phosphates involves the donation of electrons from a nucleophile to a phosphorus, leading to partial bond formation in the transition state. The dominant feature controlling nucleophilicity in these reactions is the Lewis basicity of the nucleophile. Considering its high pK$_a$ of about 13.5 (17), the 2’-oxygen of ribose is a very reactive species by itself. However, the concentration of the reactive species is very low because most of the 2’-oxygen will be protonated at a neutral pH value. In the classical case of serine proteases, the nucleophilic serine is activated in a catalytic Asp-His-Ser triad. The present study aims to shed more light on how ribonucleases deal with this problem of...
reaction is the formation of a Michaelis complex between enzyme and substrate. Starting from high resolution structures of genuine Michaelis complexes, ab initio quantum chemical calculations were used to evaluate the effect of the enzymic environment upon the intrinsic reactivity of the 2'-hydroxyl group in RNA.

**EXPERIMENTAL PROCEDURES**

**X-ray Crystallography—** All RNase T1 mutants were co-crystallized with the substrate 3'-guanosine monophosphate (3'-GMP) using the hanging drop vapor diffusion method. 1.5 mM protein and 3 mM substrate were incubated together for an hour at 20 °C prior to adding an equal volume of mother liquor containing 2 mM CaCl2, 50 mM sodium acetate, pH 4.2, and 42–52% 2-methyl-2,4-pentanediol. Data collection and refinement procedures were carried out essentially as published (18); the results are summarized in Table I. The atomic coordinates for the H40A, E58A, and corresponding double mutant of RNase T1 have been submitted to the Protein Data Bank, with respective entries 1LOW, 1LOV, 1LOY. Atomic coordinates for wild type RNase T1 and RNase A were taken from the Protein Data Bank (entries 1RGA and 1RPF, respectively).

**Charge Distribution in the Active Sites of RNase T1 and RNase A—** In a first set of computations, we analyzed the charge distribution in the Michaelis complex of wild type and mutant RNase T1, starting from high resolution crystallographic data (Table I). The models used in the calculations consisted of the 3'-GMP substrate and all amino acid side chains that make up the active site: Tyr-38, His-40, Glu-58, Arg-77, His-92, and Phe-100 (Fig. 2a). A similar analysis was done on a model of wild type RNase A that was constructed starting from the crystallographic coordinates of the complex with 3'-CMP (12), including the side chains of Glu-11, His-12, Lys-41, and His-119 and the backbone atoms of Phe-100 (Fig. 2b). The mutants of RNase A were constructed in *silico* by truncating a side chain at the Cβ atom in the wild type model. In all cases, hydrogens were added according to the protonation state of titratable groups and optimized at the Hartree Fock 3-21G level of theory, whereas heavy atoms were kept fixed at their spatial positions. As a control, each RNase T1 mutant was constructed in *silico* as well. For all these models, atomic charges were obtained using the Mulliken population analysis method (19). The Mulliken analysis was preferred to the nowadays often used CHELPG analysis (35) because the latter leads to a poorer description of atoms embedded in large systems as these atoms contribute less to the electrostatic potential at the surface considered. To validate the use of the Mulliken charge as a descriptor of nucleophilicity, the Mulliken charge distribution was calculated for four alkoxide nucleophiles attacking methyl 2,4-dinitrophenyl phosphate. Fig. 3 plots the net atomic charge on the nucleophilic oxygen versus the logarithm of experimentally obtained rate constants for the nucleophilic substitution reaction (36). The observed correlation allows for a qualitative assessment of the 2' nucleophilicity based on Mulliken charges. Whereas the charge on an oxygen atom describes its nucleophilicity, the polarity of a hydroxyl group, which may be estimated by the product of the charges on the oxygen and hydrogen atoms, describes its acidity (37–39). The higher the polarity of the 2'-OH group, the greater proton transfer to a catalytic base will be facilitated.

**Analysis of Intermolecular Hydrogen Bonds in the Nucleophile Binding Pocket—** To analyze the interactions of the 2'-nucleophile with the catalytic dyad at the quantum chemical level, the following model was constructed. Methanol was used to mimic the 2'-hydroxyl group, formate for glutamate, a methylammonium ion for lysine, and an imidazolium ion in the case of RNase T1. Ab initio calculations using the Hartree Fock, Möller Plesset 2, and density functional theory (DFT) methodology (the latter two methods partly taken into account the electronic correlation energy)

have been carried out using the split valence 6–31+G(d,p) basis set, which includes d polarization and diffuse sp functions on heavy atoms and p polarization and diffuse p functions on hydrogen atoms. The DFT B3LYP functional, which proved to be adequate for the calculation of a large variety of molecular properties (20, 21) including hydrogen bond energies (22), is a hybrid functional constructed by combining Becke’s exchange functional (23, 24) and the LYP correlation functional (25). Hydrogen atoms were optimized at each level of theory, heavy atoms being fixed at their spatial positions as observed in the crystal structure. The hydrogen bond energy is calculated as the difference between the energy of the complex and the energies of the infinitely separated partners. Basis set superposition errors were corrected for using the counterpoise method (26). All calculations were performed using methods implemented in the Gaussian98 package (27).

**RESULTS AND DISCUSSION**

**X-ray Structures of the RNase T1 Michaelis Complexes—** Table I summarizes the crystallographic details of the structures that were used as a starting point for the computational study. In each mutant structure, the 3'-GMP substrate is bound in a catalytic productive mode, as is the case for the wild type structure (8). Apart from direct interactions involving the mutated residues, all intermolecular interactions between enzyme and substrate are found to be similar in all these structures. The only striking difference among these structures is observed in the E58A complex where the imidazole side chain of His-40 has flipped about 180 °, causing it to interact with the 2'-oxygen via its Nδ atom instead of Nε as is found in the wild type structure.

**Mulliken Atomic Charges as Nucleophilicity Descriptors—** A diversity of reactivity descriptors is available to probe nucleophilicity. Working within the context of Pearson's hard and soft acids and bases principle (28, 29) at a local level (30–32), the hardness of the reacting partners in the RNase active site incites us to use DFT-based descriptors related to local hardness. As atomic charges were found to be successful in describing the reactivity of hard centers (33, 34), they were used for this purpose. In view of computational simplicity, atomic charges were obtained via the Mulliken population analysis (19). The Mulliken analysis was preferred to the nowadays often used CHELPG analysis (35) because the latter leads to a poorer description of atoms embedded in large systems as these atoms contribute less to the electrostatic potential at the surface considered. To validate the use of the Mulliken charge as a descriptor of nucleophilicity, the Mulliken charge distribution was calculated for four alkoxide nucleophiles attacking methyl 2,4-dinitrophenyl phosphate. Fig. 3 plots the net atomic charge on the nucleophilic oxygen versus the logarithm of experimentally obtained rate constants for the nucleophilic substitution reaction (36). The observed correlation allows for a qualitative assessment of the 2' nucleophilicity based on Mulliken charges. Whereas the charge on an oxygen atom describes its nucleophilicity, the polarity of a hydroxyl group, which may be estimated by the product of the charges on the oxygen and hydrogen atoms, describes its acidity (37–39). The higher the polarity of the 2'-OH group, the greater proton transfer to a catalytic base will be facilitated.

**Nucleophilicity of the 2'-Hydroxyl in the Active Sites of RNase T1 and RNase A—** We calculated the effect of a particular mutation on the Mulliken charge on the 2'-oxygen atom to identify amino acid side chains involved in nucleophile activation. Table II summarizes both the net atomic charge of the 2'-OH group as a measure of its nucleophilicity as well as the polarity of the 2'-OH group as a measure of the degree of proton transfer to the catalytic base. In a first set of calculations, we started from x-ray crystallographic data to quantify the effect of mutations in the active site of RNase T1 on the 2’ nucleophilicity. For these calculations, His-40 was double protonated in the wild type
enzyme, whereas in the E58A mutant a neutral imidazole ring was used (7). From these results it is seen that deletion of the negatively charged Glu-58 carboxylate (the catalytic base) and/or the positively charged His-40 imidazolium lowers the Mulliken charge on the 2'-oxygen atom, thus affecting its nucleophilicity. In the wild type enzyme, the 2'-atomic charge and the polarity of the 2'-hydroxyl are maximal, illustrating that both Glu-58 and His-40 contribute to nucleophile activation. In the H40A and the E58A mutants, the 2'-OH is polarized by donating a hydrogen bond to the Glu-58 carboxylate and to the His-40 imidazole, respectively. In the case of the wild type enzyme, a second interaction is present: the charged His-40 donates a hydrogen bond to the 2'-oxygen atom. These data are entirely consistent with results from protein engineering and kinetic experiments (40). It thus follows that the comparison of quantum chemical reactivity descriptors among wild type and mutant structures is a valid complementary technique to evaluate the contribution of particular side chains to catalysis.

To test if this method is also applicable when no crystal structures are available for the mutants under investigation, we constructed a number of RNase T1 mutants in silico by removing the carboxylate and/or imidazolium side chain from the wild type structure. In a second step, we calculated the effect of these in silico mutations on the 2'-oxygen atomic charge using the same approach. Each in silico single mutant displays a higher nucleophilicity than the corresponding in vivo mutant (Table II). This may be due to the fact that in wild type RNase T1 the distances from O-2' to Nε of His-40 (2.59 Å) or Oδ of Glu-58 (2.65 Å) are smaller than in the in vivo mutants, E58A (2.92 Å) and H40A (2.75 Å). The corresponding double mutant displays a higher nucleophilicity in vivo than in silico because of the presence of a water molecule that donates a

\[
{\text{Nucleophile Activation in RNases}}
\]

![Fig. 2. Michaelis complexes of RNase T1 with 3'-GMP (a) and RNase A with 3'-CMP (b). Hydrogen, carbon, nitrogen, oxygen, and phosphorus atoms are in white, gray, blue, red and orange, respectively. c, superposition of the ribose carbon atoms in RNases A (red) and T1 (blue) shows the functional similarity of the 2' binding pockets. Bases were omitted for clarity.](image)

Statistics for data collection and refinement for three mutants of RNase T1, complexed with the minimal substrate 3'-GMP.

| Data collection | H40A | E58A | H40A/E58A |
|----------------|------|------|------------|
| Space group    | P2,2,2 | P2,2,2 | P2,2,2 |
| Resolution limit, Å | 40.217 | 40.631 | 40.023 |
| Observed reflections | 7613 | 15,595 | 12,848 |
| Refinement     | 1.90 | 1.55 | 1.55 |
| Ordered water molecules | 99.31 | 99.45 | 94.48 |
| r.m.s. deviations from ideal geometry | 0.006 | 0.005 | 0.005 |
| Bond lengths, Å | 1.4 | 1.4 | 1.4 |
| Average B factor, Å² | 127.1 | 79.4 | 88.0 |

TABLE II

Nucleophilicity of the 2'-OH group, illustrated by the Mulliken atomic charge on O-2' and the polarity of the hydroxyl bond for a variety of Michaelis complexes in atomic units.

| RNase T1 | Wild type | E58A in vivo | H40A in vivo | H40A/E58A in vivo | E58A in silico | H40A in silico | H40A/E58A in silico | RNase A in silico | Wild type | H12A in silico | K41A in silico | H12A/K41A in silico |
|----------|-----------|--------------|--------------|-------------------|---------------|---------------|-------------------|------------------|-----------|--------------|--------------|-------------------|
| Mulliken charge O-2' | -0.770 | -0.712 | -0.715 | -0.702 | -0.741 | -0.724 | -0.686 | -0.799 | -0.795 | -0.765 | -0.758 |
| Polarity 2'-OH | 0.351 | 0.315 | 0.305 | 0.300 | 0.329 | 0.321 | 0.271 | 0.439 | 0.393 | 0.395 | 0.354 |
hydrogen bond to O–2'. From the in silico mutations, it is clear that the presence of the His-40 imidazolium ion is intrinsically important for the 2'-nucleophilicity, more so than the presence of the Glu-58 side chain. This is reflected by the $k_{\text{cat}}/K_m$ values of these mutants, the catalytic importance of His-40 prevailing over Glu-58 (40).

The good qualitative agreement between the in vivo and in silico results for RNase T1 justifies the employment of the in silico approach for RNase A, for which there are no mutant crystal structures available. In RNase A, removal of the Lys-41 side chain lowers the nucleophilicity more so than removal of the catalytic base His-12. The observation that each RNase A variant displays a higher 2'-nucleophilicity than its RNase T1 counterpart can be explained by a third short hydrogen bond between the 2'-OH and a nonbridging phosphoryl oxygen. This interaction is not present in the RNase T1 complex, where the phosphate group is more distant from the 2'-hydroxyl group. Nevertheless, both enzymes display a similar behavior of the 2'-nucleophilicity in response to equivalent in silico mutations (H40A versus K41A and E58A versus H12A). Also, in both cases, the double mutant has the largest impact on the reactivity of the nucleophile. Interestingly, the effect of these mutations on the charge of the 2'-oxygen atom follows the same trend as the kinetic parameter $k_{\text{cat}}/K_m$ (10, 40).

**Proton Transfer to the Catalytic Base**—Based on the observed nucleophile activation, we set out to calculate the strength of the hydrogen bond between the 2'-OH group and the catalytic base in the gas phase. This was done in the presence and absence of the charged hydrogen bond donor. Table III summarizes the results for RNase A and RNase T1. The calculated hydrogen bond strength depends on the level of theory used. From the deviation values obtained at the Hartree Fock level, it is clear that electron correlation needs to be included for a proper description of the system. The DFT and Møller-Plesset 2 levels give comparable results, confirming that the B3LYP functional is suitable for this type of studies. In the following discussion we will focus on the Møller-Plesset 2 results because these may be considered the most accurate. In wild type RNase T1, the hydrogen bond strength amounts to about 17 kcal/mol, corresponding well with the experimental value of 17.6 kcal/mol for a hydrogen bond between formate and methanol in the gas phase (41). In contrast, the analogous hydrogen bond in RNase A is much weaker (about 6.7 kcal/mol). This discrepancy can be attributed to the fact that RNase T1 features an ionic homonuclear hydrogen bond (OH–O–), whereas in RNase A we find an uncharged heteronuclear hydrogen bond (OH–N). This is also clear from the larger donor-acceptor distance in RNase A (2.90 Å) as compared with RNase T1 (2.64 Å). All the same, removal of the His-40 imidazolium in RNase T1, or the Lys-41 ammonium group in RNase A leads to a decrease in hydrogen bond strength of 3 and 4 kcal/mol, respectively. These computational results are consistent with the functional cooperativity that has been observed experimentally between the side chains of His-40 and Glu-58 in RNase T1 (40). These two residues depend on each other to perform their mutual role in catalysis. It is now clear that His-40 assists in the proton transfer from 2'-OH to Glu-58 via the donation of a charged hydrogen bond.

Taken together, these results promote a mechanism of nucleophile activation in RNA by a catalytic dyad in an RNase active site. This may well be a general feature for many ribonucleases. Human angiogenin for example, an effector of blood vessel formation, relies for catalytic activity on two histidines (His-13 and His-114) and a lysine (Lys-40), like all members of the RNase A superfamily. An analogous study on a molecular dynamics structure of angiogenin complexed with the dinucleotide CpA (42), revealed a decrease of 3 kcal/mol in the strength of the hydrogen bond between His-13 and 2'-OH upon removal of the Lys-40 side chain (data not shown). These results suggest a general mechanism for nucleophile activation in ribonucleases by a catalytic dyad composed of the catalytic base and a charged hydrogen bond donor. In the Michaelis complex, the ground state of the enzyme-catalyzed reaction, the catalytic base accepts a hydrogen bond from the 2'-OH while the cationic partner donates a hydrogen bond to the 2'-oxygen atom, thereby strengthening the first hydrogen bond. Because mutation of the dyad residues affects catalysis, rather than binding, these interactions will become significantly more important in the transition state of the catalyzed reaction. Such a mechanism is reminiscent of the two-metal-ion-assisted phosphoryl transfer catalyzed by various enzymes and ribozymes (43, 44). There, one divalent metal ion stabilizes negative charge build up on the leaving oxygen, while another is directly coordinated to the attacking oxygen. An increasing number of crystal structures of enzyme complexes, wherein the nucleophile is present in a productive configuration (8, 12, 13, 45–48), argues for a common mechanism of nucleophile activation in which a cationic group interacts directly with the attacking atom, resulting in enhanced general base catalysis. Particularly, we believe that a charged hydrogen bond donated to the 2'-oxygen atom is indispensable for efficient proton transfer to the catalytic base. In conclusion, this study demonstrates how high level ab initio computations on “experimental” X-ray structures of Michaelis complexes can lead to novel insights in enzymology. Interestingly, the onset of catalysis is already noticeable in the Michaelis complex, as is clear from the negative charge increase on the attacking nucleophile upon substrate binding.

**Table III**

|                      | HF       | B3LYP    | MP2      |
|----------------------|----------|----------|----------|
| RNase T1             |          |          |          |
| With His-40          | 13.3     | 17.4     | 16.9     |
| Without His-40       | 11.9     | 14.6     | 13.9     |
| RNase A              |          |          |          |
| With Lys-41          | 3.3      | 6.5      | 6.7      |
| Without Lys-41       | −0.5     | 1.9      | 2.7      |

*HF, Hartree Fock; MP2, Møller-Plesset 2.*

**FIG. 3.** Correlation between the Mulliken charge on the nucleophilic oxygen atom and the experimentally observed second order rate constant (38) for the attack of phenolato, p-cyanophenolate, p-chlorophenolate and p-ethoxycarboxyphenolate on methyl 2,4-dinitrophenyl phosphate ($n = 4$, $R^2 = 0.9844$).
REFERENCES

1. Eckstein, F., Schulz, H. H., Ruterjans, H, Maurer, W., and Haar, W. (1972) *Biochemistry* 11, 3507–3512
2. Usher, D. A., Benenreich, E. S., and Eckstein, F. (1972) *Proc. Natl. Acad. Sci. U. S. A.* 69, 115–118
3. Raines, R. T. (1998) *Chem. Rev.* 98, 1045–1065
4. Steyaert, J. (1997) *Eur. J. Biochem.* 247, 1–11
5. Loverix, S., and Steyaert, J. (2001) *Methods Enzymol.* 341, 305–323
6. Hill, C., Dodson, G., Heinemann, U., Saenger, W., Mitsui, Y., Nakamura, K., Borisov, S., Tischchenko, G., Polyakov, K., and Pavlovskii, S. (1983) *Trends Biochem. Sci.* 8, 364–369
7. Steyaert, J., Hallenga, K., Wyns, L., and Stanssens, P. (1990) *Biochemistry* 29, 9064–9072
8. Zegers, I., Haikal, A. F., Palmer, R., and Wyns, L. (1994) *J. Biol. Chem.* 269, 127–133
9. Cordes, F., Stariikov, E. B., and Saenger, W. (1995) *J. Am. Chem. Soc.* 117, 16365–16372
10. Messmore, J. M., Fuchs, D. N., and Raines, R. T. (1995) *J. Am. Chem. Soc.* 117, 8057–8069
11. Herschlag, D. (1994) *J. Am. Chem. Soc.* 116, 11631–11635
12. Zegers, I., Maes, D., Daotthi, M. H., Poortmans, F., Palmer, R., and Wyns, L. (1994) *Protein Sci.* 3, 2322–2339
13. Fisher, B. M., Schultz, L. W., and Raines, R. T. (1998) *Biochemistry* 37, 17386–17401
14. Wladkowski, B. D., Svensson, L. A., Sjoen, L., Ludner, J. E., and Gilliland, G. L. (1998) *J. Am. Chem. Soc.* 120, 5488–5489
15. Haydock, K., Lim, C., Brunger, A. T., and Karpus, M. (1990) *J. Am. Chem. Soc.* 112, 3826–3831
16. Sowa, G. A., Hengge, A. C., and Cleland, W. W. (1997) *J. Am. Chem. Soc.* 119, 2319–2320
17. Velikyan, I., Achariya, S., Trifonova, A., Feoddes, A., and Chattopadhyaya, J. (2001) *J. Am. Chem. Soc.* 123, 2893–2894
18. Zegers, I., Loris, R., Dehollander, G., Haikal, A. F., Poortmans, F., Steyaert, J., and Wyns, L. (1998) *Nat. Struct. Biol.* 5, 280–283
19. Mulliken, R. S. (1855) *J. Chem. Phys.* 23, 1853, 1854, 2238, 2343
20. Geerlings, P., De Proft, F., and Langenaeker, W. (1999) *Adv. Quant. Chem.* 33, 303–328
21. Koch, W., and Holthausen, M. C. (2001) *A Chemist's Guide to Density Functional Theory*, Wiley-VCH, Weinheim, Germany
22. Becke, A. D. (1993) *Chem. Phys. Phys.* 98, 5648–5652
23. Becke, A. D. (1996) *Chem. Phys.* 194, 1040–1046
24. Lee, C., Yang, W., and Parr, R. G. (1988) *Phys. Rev. B* 37, 785–789
25. Boys, S. F., and Bernardi, F. (1970) *Mol. Phys.* 10, 553–566
26. Frisch, M. J., Trucks, G. W., Schlegel, H. B., Scuseria, G. E., Robb, M. A., Cheeseman, J. R., Zakrzewski, V. G., Montgomery, J. A., Jr., Stratmann, R. R., Burant, J. C., Dapprich, S., Millam, J. M., Daniels, A. D., Kudin, K. N., Strain, M. C., Farkas, O., Tomasi, J., Barone, V., Cossi, M., Cammi, R., Mennucci, B., Pomelli, C., Adamo, C., Clifford, S., Ochterski, J., Petersson, G. A., Ayala, P. Y., Cui, Q., Morokuma, K., Malick, D. K., Rabuck, A. D., Raghavachari, K., Foresman, J. B., Cioslowski, J., Ortiz, J. V., Baboul, A. G., Stefanov, B. B., Liu, G., Liashenko, A., Piskorz, P., Komaromi, I.,omperts, R., Martin, R. L., Fox, D. J., Keith, T., Al-Laham, M. A., Peng, C. Y., Nanayakkara, A., Gonzalez, C., Challacombe, M., Gill, P. M. W., Johnson, B. G., Chen, W., Wong, M. W., Andres, J. L., Head-Gordon, M., Repluge, E. S., and Pople, J. A. (1998) *Gaussian 98*, Gaussian Inc., Pittsburgh, PA
27. Pearson, R. G. (1963) *J. Am. Chem. Soc.* 85, 3533–3539
28. Parr, R. G., and Yang, W. (1989) *Density-Functional Theory of Atoms and Molecules*, pp. 87–104, Oxford University Press, New York
29. Mendez, F. and Gazquez, J. L. (1994) *J. Am. Chem. Soc.* 116, 9288–9301
30. Gazquez, J. L., and Mendez, F. (1994) *J. Phys. Chem.* 98, 4591–4593
31. Gazquez, J. L., and Mendez, F. (1994) *J. Phys. Chem.* 98, 4591–4593
32. Geerlings, P., and De Proft, F. (2000) *Int. J. Quantum Chem.* 80, 227–235
33. Langenaeker, W., De Proft, F., and Geerlings, P. (1995) *J. Phys. Chem.* 99, 6424–6431
34. Chattaraj, P. K., Gomez, B., Chamorro, E., Santos, J., and Fuentealba, P. (1994) *J. Phys. Chem.* A 98, 8815–8820
35. Breneman, C. M., and Wiberg, K. B. (1999) *J. Comput. Chem.* 11, 361–373
36. Kirby, A. J., and Younas, M. (1970) *J. Chem. Soc. B.* (6), 1165–1172
37. Datka, J., Geerlings, P., Mortier, W., and Jacobs, P. (1985) *J. Phys. Chem.* 89, 3483–3488
38. Langenaeker, W., Coussement, N., De Proft, F., and Geerlings, P. (1994) *J. Phys. Chem.* 98, 3010–3014
39. Vos, A. M., Nulens, K. H. L., De Proft, F., Schoonheydt, R. A., and Geerlings, P. (2002) *J. Phys. Chem.* 106, 2026–2034
40. Steyaert, J., and Wyns, L. (1993) *J. Mol. Biol.* 229, 770–781
41. Ment-Ner, M., and Sieck, L. W. (1986) *J. Am. Chem. Soc.* 108, 7525–7529
42. Sushch, M., Szabo, A., and Vaidhivaswara, S. (2001) *Proteins Struct. Funct. Genet.* 45, 30–39
43. Steitz, T. A., and Steitz, J. A. (1993) *Proc. Natl. Acad. Sci. U. S. A.* 90, 8057–8062
44. Lott, W. B., Pontius, B. W., and Von Hippel, P. (1998) *Proc. Natl. Acad. Sci. U. S. A.* 95, 542–547
45. Guillemet, V., Laphorn, A., and Mauguen, Y. (1993) *FEBS Lett.* 330, 137–140
46. Duggal, S. D., Slepy, S. P., Hill, C. P., Dodson, E. J., Dodson, G., and Moody, P. C. E. (1995) *Nature* 373, 264–268
47. Essen, L.-O., Perissi, O., Cheung, R., and Williams, R. L. (1996) *Nature* 380, 595–602
48. Numata, T., and Kimura, M. (2001) *J. Biochem. (Tokyo)* 130, 621–626
