An ab Initio Molecular Orbital Study of the First Step of the Catalytic Mechanism of Thymidylate Synthase: The Michael Addition of Sulfur and Oxygen Nucleophiles

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Received June 29, 1995®

Ab initio molecular orbital calculations are used to study the Michael additions of sulfur and oxygen anionic nucleophiles to acrolein. The energetic and structural results of these calculations provide insight into the formation of the covalent enzyme–substrate complex of thymidylate synthase with dUMP, as well as the stereospecificity of Michael additions of heteroanionic nucleophiles. In addition, we discuss the interesting effect that substituents of the nucleophile (hydrogen vs methyl) have on the energy of reaction.

Introduction and Background

Thymidylate synthase (TS) catalyzes the reductive methylation of 2′-deoxyuridylate (dUMP) to form 2′-deoxythymidylate (dTMP) using 5,10-methylenetetrahydrofolate as a methylene donor and hydride reductant. This reaction is part of the single *de novo* pathway for the synthesis of dTMP. Consequently, the design of new anticancer drugs has been targeted at the inhibition of TS. A variety of folate-derived inhibitors of TS have been studied.[1]

The basic features of the catalytic mechanism of TS have been established.[2] The first step of the catalytic mechanism of TS after formation of the binary complex is the Michael addition of a catalytic cysteine residue (Cys198)[3] to dUMP. The existence of the covalent enzyme–substrate complex has been established in crystal structures of TS with dUMP[4] and TS with 5-fluoro-dUMP,[5] by ^{19}F NMR,[6] and by isotopic-labeling studies.[7]

Michael additions are proposed as part of a number of enzymatic mechanisms[8] and are also important reactions in organic synthesis.[9] Thiols are known to act as nucleophiles in Michael additions.[10–13] In recent years, the stereochemistry of Michael additions of heteronucleophiles has been examined in a number of systems. Mohrig et al. have studied the addition of oxygen and sulfur nucleophiles to ethyl crotonate.[11] These reactions are stereoselective with anti addition of the proton. Kamimura et al. studied the addition of thiols to nitro olefins and also observed anti selectivity.[12a] Anti specific Michael additions of thiols to α,β-unsaturated carboxylic acids have observed by Miyata et al.[12c]

Michael additions have also been studied using quantum mechanical calculations.[12,14] Wong et al. studied the 1,2- and 1,4-additions of cyanide to acrolein in an effort to better understand the factors controlling the regiochemical outcome of these additions.[14a] Recently, Pardo et al. examined the addition of ammonia to three Michael acceptors to better understand the nucleophilic additions of DNA with common Michael acceptors.[14e] Also, various intermediates of Michael additions have been examined using quantum mechanical calculations[12] to explain the stereochemistry of proton addition to the enolate. Kamimura et al. proposed that the preferred conformation of the enolate places the nucleophile in a position to block the syn (to the nucleophile) face of the enolate, resulting in anti addition of the proton.[12a,b] In contrast, Miyata et

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® Abstract published in *Advance ACS Abstracts,* November 15, 1995.

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al. proposed that the enolate carbanion pyramidalizes away from the nucleophile due to a stereoelectronic effect of the neighboring sulfur group,[12c] similar to previous suggestions relating stereoselectivity to pyramidalization of an sp^2 carbon.[15] Proton addition occurs anti to the nucleophile due to better orbital overlap with the pyramidalized enolate carbanion.

In this paper, we examine the potential energy surface of some simple Michael additions that model the formation of the covalent enzyme–substrate complex of TS using ab initio molecular orbital calculations. This study presents an interesting comparison to a previous study on the addition of sulfur nucleophiles to carbonyl compounds, important in the enzymatic hydrolysis of peptides by cysteine proteases. In that study, it was demonstrated that tetrahedral intermediates for the additions of HS^- to formamide and formaldehyde do not exist.[16] We also discuss the interesting effect that changing a proton attached to the nucleophilic center to a methyl group has on the energy of reaction.

Computational Procedure

Ab initio molecular orbital calculations were performed with restricted Hartree–Fock (RHF) theory using the GAUSSIAN 92 programs.[17] The 3-21+G(d), 4-31+G(d), and 6-31+G(d) basis sets[18–20] were employed for the RHF geometry optimizations. Harmonic vibrational frequencies were calculated to confirm the nature of all stationary points. To account for the effects of electron correlation, single-point energy evaluations were performed using the 6-31+G(d) basis set and second-order Møller–Plesset perturbation theory.[21] For model A, all stationary points were reoptimized at the MP2/6-31+G(d) level.[22] The energies reported in this study were not corrected for basis set superposition error[23] due to the uncertainty involved with this type of correction.[24] In order to investigate the "methyl effect", the ΔE_{rxn} for the 1,2-additions of oxygen and sulfur nucleophiles to formaldehyde were investigated. The CS bond was constrained to 1.90 Å for the additions of HS^- and CH_3S^- because intermediates do not exist for these reactions at the RHF level.[16,25] The structures were optimized at the RHF/6-31+G(d) level, and single points were performed at the MP2/6-31+G(d) level.

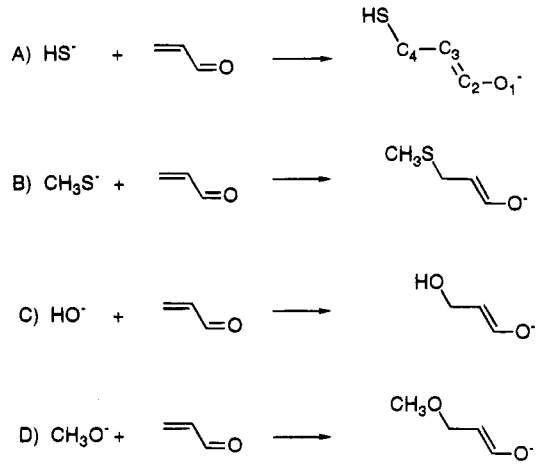

Figure 1. Four model systems investigated.

Results

We were interested in the energetics and structures of the stationary points on the potential energy surface for the formation of the covalent enzyme–substrate complex between TS and dUMP. In order to reduce the size of the system such that quantum mechanical calculations could be employed, four model systems were chosen (Figure 1). Acrolein was picked as a model for the uracil of dUMP. HS^- and CH_3S^- were chosen as models for the catalytic cysteine (Cys198). HO^- and CH_3O^- were chosen as models for serine, which has been substituted for the Cys198 using site-directed mutagenesis.[26] The stationary points on the potential energy surface of each model system were located. The acrolein was kept in the s-trans conformation in order to mimic uracil.

The total energies for all stationary points in models A, B, C, and D are given in Table 1, and the relative energies for each model with respect to the infinitely separated reactants are given in Table 2. Two ion–dipole complexes were located for each model system (Figure 2). The nucleophile interacts with a hydrogen attached to the terminal carbon of acrolein. The complexes involving the hydrogen cis to the CC single bond are the lowest in energy (**2**, **4**, **6**, **8**). These results are consistent with calculations of Wong et al. on the Michael addition of cyanide to acrolein.[14a]

A single transition structure for each model system was located (Figure 3) in which the conformation about the forming CX bond (X = O, S) is near eclipsed. Transition structures corresponding to other conformations about the forming CX bond were not located. In each case, the nucleophile rotates back to the eclipsed conformation. At the RHF/6-31+G(d) level, the forming CS bond lengths are 2.19 and 2.30 Å in **9** and **10**, respectively, and the forming CO bond lengths are 2.27 and 2.18 Å in **11** and **12**, respectively. **12** is very similar in structure to the transition state for the addition of CH_3O^- to 2-methyl-

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Table 1. Total Energies of Reactants, Ion–Dipole Complexes, Transition Structures and Products for the Michael Additions of HS^-, CH_3S^-, HO^-, and CH_3O^- with Acrolein

structure	RHF/3-21+G(d)	RHF/4-31+G(d)	RHF/6-31+G(d)	IMF	ZPE	MP2/6-31+G(d)[a]
acrolein (s-trans)	−189.719 99	−190.586 69	−190.769 92		41.6	−191.322 85
HO^-	−74.995 74	−75.307 68	−75.376 42		5.7	−75.587 78
HS^-	−396.265 72	−397.705 34	−398.106 89		4.0	−398.229 53
CH_3O^-	−113.803 43	−114.303 83	−114.411 12		24.2	−114.743 62
CH_3S^-	−435.070 82	−436.687 97	−437.126 93		24.3	−437.384 47
1	−586.003 90	−588.308 57	−588.892 70		46.1	−589.571 53
2	−586.007 02	−588.310 18	−588.894 58		46.5	−589.573 68
3	−624.809 99	−627.291 06	−627.912 83		66.5	−628.727 75
4	−624.812 67	−627.292 96	−627.914 71		66.7	−628.728 88
5	−264.754 70	−265.925 86	−266.177 88		48.2	−266.944 42
6	−264.760 31	−265.930 51	−266.182 55		48.8	−266.949 13
7	−303.537 06	−304.909 85	−305.208 94		66.8	−306.097 22
8	−303.564 49	−304.922 44	−305.212 91		67.2	−306.101 33
9	−585.984 60	−588.281 63	−588.866 32	312.3i	47.0	−589.571 14
10	−624.799 31	−627.273 22	−627.895 07	289.8i	67.1	−628.736 91
11	−264.744 97	−265.917 86	−266.169 89	172.5i	48.6	−266.942 38
12		−304.910 76	−305.201 19	161.5i	67.5	−306.101 77
13	−585.989 24	−588.284 25	−588.869 83		47.8	−589.576 44
14	−624.808 47	−627.279 55	−627.902 32		67.7	−628.745 38
15	−624.805 18	−627.276 53	−627.899 58		67.5	−628.740 54
16	−624.805 89	−627.276 98	−627.899 75		67.6	−628.742 49
17	−264.782 34	−265.961 31	−266.213 91		51.5	−266.981 39
18	−303.586 93	−304.944 24	−305.235 09		70.4	−306.132 36
19	−303.579 89	−304.940 80	−305.231 68		70.2	−306.127 94
20	−303.585 34	−304.942 82	−305.233 64		70.4	−306.130 52

[a] Single-point energy calculation on RHF/6-31+G(d) geometry.

Table 2. Relative Energies of Reactants, Ion–Dipole Complexes, Transition Structures, and Intermediates for the Michael Additions of HS^-, CH_3S^-, HO^-, and CH_3O^- to Acrolein. Energies Are in kcal/mol

structure	RHF/3-21+G(d)	RHF/4-31+G(d)	RHF/6-31+G(d)	MP2/6-31+G(d)[a]	MP2/6-31+G(d)[a] + ZPE[b]
HS^- + acrolein	0.0	0.0	0.0	0.0	0.0
2	−13.4	−11.4	−10.1	−13.3	−12.4
9 (TS)	0.7	5.5	7.6	−11.7	−10.3
13	2.2	3.9	5.4	−15.1	−12.9
CH_3S^- + acrolein	0.0	0.0	0.0	0.0	0.0
4	−13.7	−11.5	−11.2	−13.5	−12.7
10 (TS)	−5.3	0.9	1.1	−18.5	−17.3
14	−11.0	−3.1	−3.4	−23.9	−22.1
15	−9.0	−1.2	−1.7	−20.8	−19.2
16	−9.4	−1.5	−1.8	−22.0	−19.9
HO^- + acrolein	0.0	0.0	0.0	0.0	0.0
6	−28.0	−22.7	−22.7	−24.1	−22.6
11 (TS)	−18.3	−14.7	−14.8	−19.8	−18.5
17	−42.5	−42.0	−42.4	−44.3	−41.5
CH_3O^- + acrolein	0.0	0.0	0.0	0.0	0.0
8	−25.2	−20.0	−19.9	−21.9	−20.5
12 (TS)		−12.7	−12.6	−22.2	−20.5
18	−39.3	−33.7	−33.8	−35.8	−31.2
19	−34.9	−31.6	−31.7	−33.6	−29.2
20	−38.3	−32.8	−32.9	−34.8	−30.2

[a] Single-point energy evaluation using RHF/6-31+G(d)-optimized geometry. [b] Zero-point energy corrections obtained from unscaled RHF/6-31+G(d) frequency calculations.

acrolein.[14b] **9** was reoptimized at the MP2/6-31+G(d) level of theory (Figure 3).[22] The forming CS bond is 2.64 Å, 0.45 Å longer than in the RHF/6-31+G(d) transition structure.

All intermediates are shown in Figure 4. A single intermediate was located for models A and C. **13** and **17** are in eclipsed conformations. Three intermediates, corresponding to conformations about the newly formed CX bond (X = O, S), were located for models B and D. The most stable structures are **14** and **18**, which are gauche conformations. **15** and **19** are also gauche conformations, while **16** and **20** are staggered conformations. These results are in accord with other calculated structures for intermediates of Michael additions (Table 3).[12] C_3 is negatively charged in all the intermediates (for numbering system see Figure 1). **14** and **18** are most stable because the electron–electron repulsion between the nucleophile's lone pairs, and the negative charge on C_3 is minimized in this conformation. This phenomena has been called the endo alkoxy effect.[12a,b]

Discussion

Energy of Reaction. In this paper ΔE_{rxn} is defined as the difference between the energies of the intermediate and the infinitely separated reactants. The ΔE_{rxn} for models C and D, which have oxygen nucleophiles, are relatively independent of the level of theory at which they are calculated. The large exothermicities of these additions are in accord with other calculations of gas-phase nucleophilic additions of oxygen nucleophiles[27] and are consistent with stabilization of charge by delocalization.

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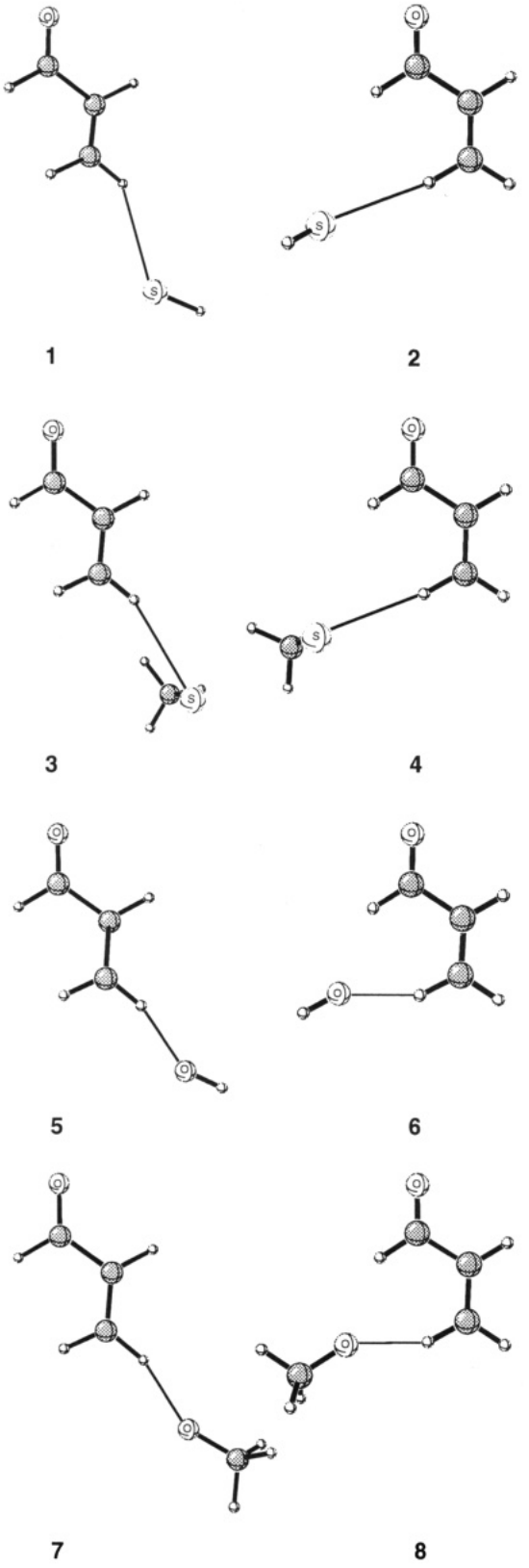

Figure 2. RHF/6-31+G(d) structures of the ion−dipole complexes for models A, B, C, and D.

In contrast, the ΔE_{rxn} for models A and B, which have sulfur nucleophiles, are more dependent on the level of theory employed. At the RHF/6-31+G(d) level, ΔE_{rxn} for models A and B are 5.4 and −3.4 kcal/mol, respectively, compared to −12.9 and −22.1 kcal/mol at the MP2/6-31+G(d)//RHF/6-31+G(d) + ZPE level. An ~18 kcal/mol change in ΔE_{rxn} was unexpected, but has important implications with regard to the transition structures for models A and B, which will be discussed later.

It is not surprising that the ΔE_{rxn} for models C and D are more exothermic than for models A and B. In models C and D, the gain of a new σ_{CO} bond and delocalization of the charge over the whole molecule compensates for the lose of a π_{CO} bond, yielding a highly exothermic reaction. The polarizability of sulfur results in the nucleophiles in models A and B being more stable than the oxygen nucleophiles in models C and D. Experimentally, sulfur's increased polarizability is manifested in the lower pK_as[28] and gas-phase acidities[29] of thiols compared to alcohols. The addition of the sulfur nucleophiles with acrolein also delocalizes the charge over the whole molecule. In this case, a π_{CO} bond is lost and σ_{CS} bond is gained. On the basis of bond energies, formation of this intermediate requires ~12 kcal/mol in models A and B.[30] In studying the addition of HS^- to formaldehyde and formamide, Howard and Kollman explained the lack of an intermediate using this simple bond energy argument.[16] The formation of intermediates in models A and B must be the result of the extra stabilization of charge by delocalization that is not possible in carbonyl additions.

The Methyl Effect. An interesting substituent effect on the ΔE_{rxn} is observed. The ΔE_{rxn} for model C is 10.3 kcal/mol more negative than that for model D at the MP2/6-31+G(d)//RHF/6-31+G(d) + ZPE level. This energy difference varies little with the level of theory. The 10 kcal/mol stabilization by the methyl group correlates well with the difference in the gas-phase acidities (GPA) of water and methanol. Methanol has a GPA of 379.2 kcal/mol compared to a GPA of 390.8 for water,[29] giving a ΔGPA[31] of −11.6 kcal/mol. A methyl group stabilizes the anion more than the proton. In the intermediates, the anion is delocalized over the whole molecule and the charge is concentrated at O_1 and C_3. The difference between a methyl group and a proton is minimal in the intermediates with regard to stabilizing the charge. This change in charge localization is reflected in the change in the CO bond length (RHF/6-31+G(d)) going from 1.33 Å in methoxide to 1.38 Å in the intermediate **18**, which is similar to the CO bond length in methanol (1.40 Å) at the same level of theory. The stabilization of the oxygen nucleophile by the methyl group lowers the ΔE_{rxn} for model D compared to model C.

The effect of the methyl group on the ΔE_{rxn} in models C and D is the opposite of that in models A and B. At the MP2/6-31+G(d)//RHF/6-31+G(d) + ZPE level the ΔE_{rxn} for model B is 9.2 kcal/mol more negative than that for model A. Again this difference correlates well with the difference in the gas-phase acidities of hydrogen sulfide and methanethiol. Hydrogen sulfide has a GPA of 353.4 and methanethiol has a GPA of 359.0,[29] giving a ΔGPA of 5.6 kcal/mol. The absolute ΔGPA (sulfur) is half the absolute ΔGPA (oxygen), reflecting sulfurs ability to stabilize charge regardless of the substituent attached

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(30) Bond strengths C−S, 66.36 kcal/mol; C−O 90.99 kcal/mol: Lovering, E. G; Laidler, K. J. *Can. J. Chem.* **1960**, *38*, 2367. C=O, 169 kcal/mol: Berry, R. S.; Rice, S. A.; Ross, J. *Physical Chemistry Part 2 [Matter in Equilibrium: Statistical Mechanics and Thermodynamics]*; Wiley: New York, 1980; p 564.

(31) ΔGPA = GPA(methyl) − GPA(hydrogen).

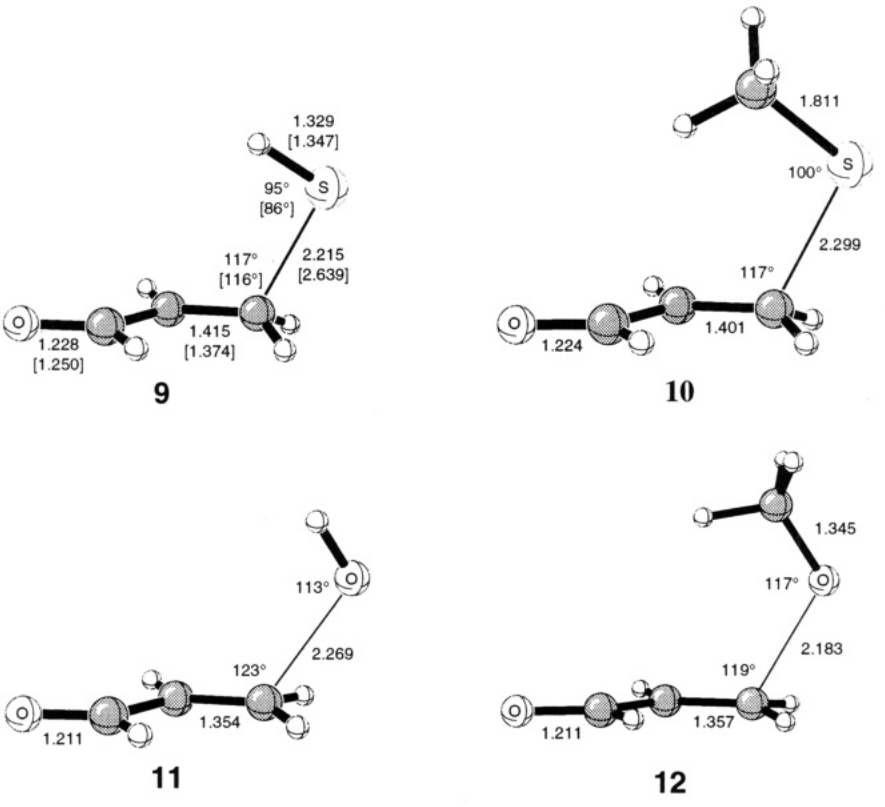

Figure 3. RHF/6-31+G(d) [MP2/6-31+G(d)] transition structures for the Michael additions of HO^-, MeO^-, HS^- and MeS^- to acrolein.

to it. In this case the methyl group destabilizes the sulfur nucleophile resulting in a larger ΔE_{rxn} for model B relative to model A.

In order to determine if the methyl effect is observable in other additions of anionic nucleophiles, we examined the addition of the four nucleophiles to formaldehyde. For the addition of the oxygen nucleophiles, the ΔE_{rxn} is 2.4 kcal/mol more exothermic for the addition of HO^- at the MP2/6-31+G(d)//RHF/6-31+G(d) level, similar but smaller in magnitude than for the 1,4-additions. With the CS bond constrained to 1.9 Å the ΔE_{rxn} is 10.0 kcal/mol more exothermic for the addition of CH_3S^- than for the addition of HS^- to formaldehyde at the MP2/6-31+G(d)//RHF/6-31+G(d) level, similar to the 1,4-additions. The stabilization of oxygen nucleophiles and the destabilization of sulfur nucleophiles by methyl groups affects the ΔE_{rxn} for additions of anionic nucleophiles but is more dramatic for the 1,4-additions studied.

Activation Energies. The activation energies, defined here as the difference between the energies of the ion–dipole complex and the transition structure, are very small for these reactions. At the RHF/6-31+G(d) level, the activation energies for models C and D are 7.9 and 7.3 kcal/mol, respectively. Inclusion of electron correlation at the MP2/6-31+G(d)//RHF/6-31+G(d) + ZPE level lowers the barrier for model C to 4.1 kcal/mol, and there is no barrier for model D. Low or zero activation barriers are consistent with other calculations of gas-phase nucleophilic additions of oxygen nucleophiles.[27,32]

The activation barriers for models A and B are 17.7 and 12.3 kcal/mol, respectively, at the RHF/6-31G(d) level. At the MP2/6-31+G(d)//RHF/6-31+G(d) + ZPE level, the activation barriers for models A and B are 2.1 and −4.6 kcal/mol, respectively. The negative activation barrier for model B is an artifact of the effect of electron correlation on the ΔE_{rxn} for models A and B. The inclusion of electron correlation results in a ~18 kcal/mol change in the ΔE_{rxn} for models A and B. On the basis of the energy difference between minima (the ion–dipole complex and the product), models A and B are endothermic at the RHF level. According to the Hammond postulate,[33] the transition structure should be late and resemble the product more than the reactants. This is readily apparent by the very short forming CS bond lengths in **9** and **10** (~2.2 Å). At the MP2 level, the reaction becomes exothermic. The transition structure should become earlier, corresponding to a longer forming CS bond. A single-point calculation is only valid if the geometry of the molecule does not change appreciably at the computational level that the single-point calculation is being performed. In models A and B studied, this is not true for the transition structures. The stationary points for model A were reoptimized at the MP2/6-31+G(d) level. The forming CS bonds in **9** lengthens to 2.64 Å with the corresponding changes in geometry. The transition structure is much earlier, in accordance with the Hammond postulate.[33] The activation barrier for model A is 4.5 kcal/mol at the MP2/6-31+G(d) + ZPE level. The inclusion of electron correlation in optimization of the stationary points increases the activation barrier by 2.9 kcal/mol. It is expected that the activation barrier for model B would also increase with MP2/6-31+G(d) optimization, giving an activation barrier of ~0.5 kcal/mol, similar to that of model D.

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Table 3. Selected Geometric Parameters for Optimized Ion–Dipole Complexes, Transition Structures, and Intermediates for the Michael Additions of HS^-, CH_3S^-, HO^-, and CH_3O^- to Acrolein. X Is the Nucleophile Heteroatom (O, S) and Y Is the Nucleophile Substitent Atom (H, C) Connected to X

no.	basis set	O_1-C_2	C_2-C_3	C_3-C_4	C_4-X	X–Y	$O_1-C_2-C_3$	$C_2-C_3-C_4$	C_3-C_4-X	C_4-X-Y	$C_2-C_3-C_4-X$	C_3-C_4-X-Y
1	RHF/3-21+G(d)	1.230	1.456	1.328	3.688	1.337	125.4	120.8	144.6	123.5	−177.2	−65.8
	RHF/4-31+G(d)	1.197	1.465	1.326	3.715	1.335	125.4	120.8	144.2	124.2	−177.4	−65.9
	RHF/6-31+G(d)	1.198	1.467	1.330	3.722	1.337	125.4	120.8	144.2	123.8	−177.3	−65.9
2	RHF/3-21+G(d)	1.232	1.468	1.327	3.744	1.337	122.7	120.7	113.8	115.5	2.9	−95.3
	RHF/4-31+G(d)	1.199	1.474	1.325	3.787	1.335	122.9	121.0	114.6	117.3	3.5	−95.4
	RHF/6-31+G(d)	1.201	1.475	1.328	3.816	1.338	123.0	120.9	115.5	113.6	2.9	−91.8
	MP2/6-31+G(d)	1.241	1.471	1.348	3.602	1.350	122.5	119.9	108.6	124.9	4.4	−103.8
3	RHF/3-21+G(d)	1.229	1.457	1.329	3.667	1.847	125.5	120.8	136.2	86.5	176.5	−100.1
	RHF/4-31+G(d)	1.197	1.465	1.327	3.720	1.829	125.5	120.8	137.7	86.3	176.2	−101.7
	RHF/6-31+G(d)	1.198	1.467	1.330	3.754	1.833	125.5	120.8	138.9	88.0	175.9	−99.1
4	RHF/4-31+G(d)	1.199	1.474	1.326	3.786	1.829	123.0	120.9	115.0	110.3	−0.7	93.6
	RHF/6-31+G(d)	1.201	1.475	1.328	3.806	1.832	123.0	120.9	115.5	114.9	−1.9	92.5
5	RHF/3-21+G(d)	1.235	1.447	1.331	2.807	0.952	126.5	121.0	132.0	135.4	180.0	0.0
	RHF/4-31+G(d)	1.200	1.458	1.332	2.889	0.950	126.3	121.0	135.4	156.4	180.0	0.0
	RHF/6-31+G(d)	1.202	1.460	1.335	2.900	0.949	126.3	121.0	135.8	154.5	180.0	0.0
6	RHF/3-21+G(d)	1.240	1.469	1.330	2.879	0.970	122.3	119.1	102.8	162.5	0.0	180.0
	RHF/4-31+G(d)	1.206	1.476	1.327	2.955	0.949	122.2	119.9	102.8	160.8	0.0	180.0
	RHF/6-31+G(d)	1.207	1.478	1.330	2.957	0.949	122.2	119.9	102.4	160.6	0.0	180.0
7	RHF/3-21+G(d)	1.235	1.449	1.336	2.725	1.395	126.4	121.5	125.4	108.8	−178.8	−97.1
	RHF/4-31+G(d)	1.200	1.459	1.332	2.888	1.330	126.2	121.1	127.4	108.2	−179.1	−106.7
	RHF/6-31+G(d)	1.201	1.462	1.334	2.992	1.339	126.1	120.9	136.0	158.7	180.0	0.0
8	RHF/3-21+G(d)	1.239	1.469	1.329	2.887	1.412	122.3	119.3	102.4	160.7	0.0	180.0
	RHF/4-31+G(d)	1.205	1.475	1.327	2.984	1.339	122.4	120.0	103.5	158.6	0.0	180.0
	RHF/6-31+G(d)	1.206	1.477	1.330	2.988	1.340	122.3	120.0	103.2	158.5	0.0	180.0
9	RHF/3-21+G(d)	1.265	1.396	1.407	2.277	1.328	128.1	121.3	115.1	93.4	−93.7	17.6
	RHF/4-31+G(d)	1.227	1.401	1.416	2.191	1.327	128.8	121.1	116.5	95.0	−94.4	27.2
	RHF/6-31+G(d)	1.228	1.406	1.415	2.215	1.329	128.7	121.1	116.7	95.1	−94.6	28.8
	MP2/6-31+G(d)	1.250	1.434	1.374	2.639	1.347	126.6	120.4	116.0	85.5	−91.0	23.4
10	RHF/3-21+G(d)	1.259	1.406	1.390	2.387	1.819	127.7	121.3	114.7	96.7	−93.8	13.4
	RHF/4-31+G(d)	1.223	1.409	1.401	2.282	1.807	128.4	121.3	116.5	99.7	−96.1	31.7
	RHF/6-31+G(d)	1.224	1.413	1.401	2.299	1.811	128.4	121.3	116.5	100.0	−96.1	28.7
11	RHF/3-21+G(d)	1.244	1.429	1.352	2.260	0.973	126.7	120.6	125.5	129.4	−88.5	−3.6
	RHF/4-31+G(d)	1.209	1.437	1.352	2.267	0.951	127.0	120.5	122.8	113.2	−88.5	−0.4
	RHF/6-31+G(d)	1.211	1.440	1.354	2.269	0.950	127.0	120.4	122.9	113.4	−88.6	−0.1
12	RHF/4-31+G(d)	1.210	1.436	1.355	2.181	1.344	127.1	120.6	119.0	117.1	−90.2	9.6
	RHF/6-31+G(d)	1.211	1.438	1.357	2.183	1.345	127.1	120.5	119.1	117.1	−90.5	11.1
13	RHF/3-21+G(d)	1.249	1.381	1.479	1.902	1.326						
	RHF/4-31+G(d)	1.243	1.379	1.472	1.911	1.325	129.9	121.8	116.4	97.0	105.4	36.8
	RHF/6-31+G(d)	1.244	1.381	1.475	1.909	1.327	129.9	121.8	116.5	97.2	106.8	38.2
	MP2/6-31+G(d)	1.276	1.396	1.466	1.915	1.341	129.5	120.9	116.2	94.2	101.5	38.6
14	RHF/3-21+G(d)	1.296	1.365	1.487	1.885	1.811	129.4	122.4	115.9	100.2	115.9	55.6
	RHF/4-31+G(d)	1.245	1.376	1.477	1.887	1.803	130.0	122.3	117.1	101.1	108.1	54.9
	RHF/6-31+G(d)	1.247	1.379	1.480	1.887	1.807	130.0	122.3	117.3	101.2	108.7	54.2
15	RHF/3-21+G(d)	1.294	1.366	1.492	1.869	1.820	130.0	122.2	112.7	100.1	112.7	−172.8
	RHF/4-31+G(d)	1.244	1.377	1.480	1.872	1.809	130.0	122.2	114.0	100.2	104.6	−173.1
	RHF/6-31+G(d)	1.245	1.379	1.483	1.874	1.813	129.9	122.2	144.0	100.3	105.2	−172.8
16	RHF/3-21+G(d)	1.293	1.367	1.487	1.897	1.811	129.1	122.7	166.9	100.2	92.8	−46.5
	RHF/4-31+G(d)	1.243	1.379	1.477	1.897	1.803	129.8	122.7	118.2	100.7	95.0	−47.9
	RHF/6-31+G(d)	1.245	1.381	1.480	1.897	1.807	129.8	122.7	118.3	100.9	95.9	−47.4
17	RHF/3-21+G(d)	1.297	1.360	1.479	1.512	0.965	129.5	121.9	113.5	107.0	99.4	11.8
	RHF/4-31+G(d)	1.244	1.380	1.483	1.444	0.948	130.1	122.5	114.5	105.5	106.0	20.0
	RHF/6-31+G(d)	1.245	1.382	1.485	1.444	0.948	130.0	122.5	114.4	105.4	106.5	20.3
18	RHF/3-21+G(d)	1.293	1.367	1.482	1.498	1.436	129.5	122.4	116.2	115.6	108.3	54.8
	RHF/4-31+G(d)	1.246	1.375	1.482	1.444	1.384	130.3	122.7	117.1	115.2	108.1	62.2
	RHF/6-31+G(d)	1.248	1.378	1.483	1.444	1.385	130.2	122.7	117.1	115.1	108.4	62.3
19	RHF/3-21+G(d)	1.292	1.367	1.474	1.497	1.433	129.4	122.0	113.2	114.0	102.3	−177.9
	RHF/4-31+G(d)	1.246	1.376	1.476	1.442	1.378	130.1	122.2	114.0	113.6	102.7	−178.0
	RHF/6-31+G(d)	1.247	1.378	1.478	1.442	1.378	130.1	122.1	114.0	113.5	103.2	−178.0
20	RHF/3-21+G(d)	1.290	1.369	1.482	1.503	1.434	129.2	122.0	117.1	115.5	90.8	−47.5
	RHF/4-31+G(d)	1.245	1.378	1.482	1.446	1.381	130.0	122.3	117.9	114.7	95.6	−57.3
	RHF/6-31+G(d)	1.246	1.380	1.484	1.447	1.382	130.0	122.2	117.9	114.7	95.9	−57.4

Stereochemistry of Michael Additions. Molecular orbital calculations have been used to explain the stereochemistry of Michael additions of heteronucleophiles. This is the first study to locate all intermediates, although an attempt has been made to describe the rotational profile about the CX_{nuc} bond using MNDO calculations and constrained optimizations.[12a,b] Two rationales have been proposed to explain the preference for anti addition. The structure for the intermediate obtained by Kamimura et al. placed the nucleophile over the syn face of the enolate, leading to the proposal that anti addition results because only the anti face of the enolate is available to the proton.[12a,b] Kamimura et al. called this the "endo alkoxy effect". Miyata et al. optimized an intermediate in roughly the same conformation. They found significant pyramidalization of the negatively charged C_3 away from the substituent.[12c] Pyramidalization was proposed to occur due to a stereoelectronic effect of the neighboring sulfur group.[12c] Proton addition occurs in an anti fashion because the anti face of the enolate provides better overlap with the proton due to the pyramidalization of C_3. Our study indicates

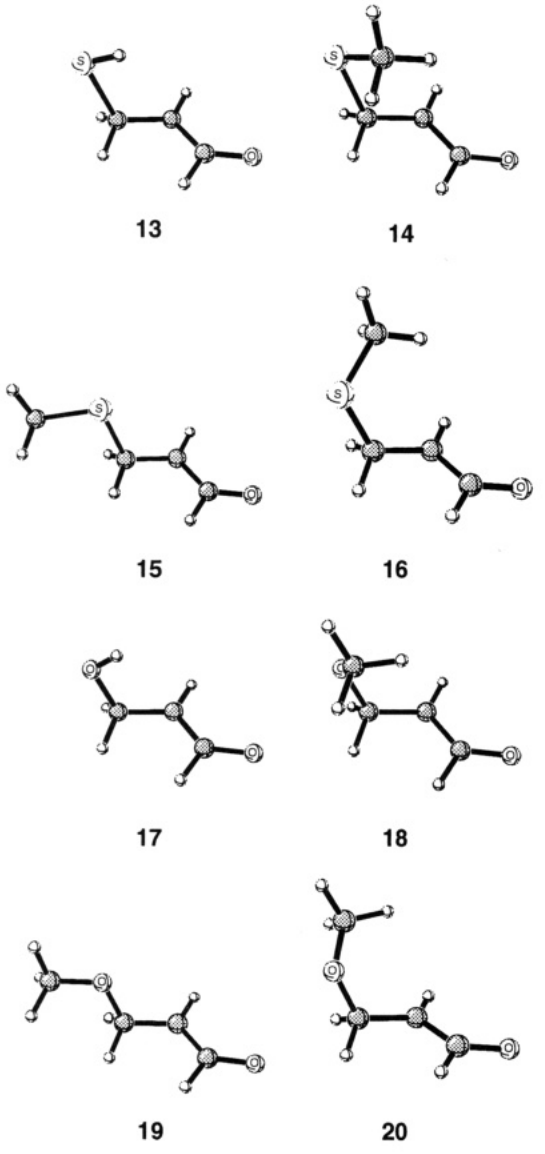

Figure 4. RHF/6-31+G(d) structures of the intermediates for models A, B, C, and D.

that the gauche conformations in models B and D are the two lowest energy conformations. In both cases, the syn face is effectively blocked by the nucleophile. Pyramidalization of C_3 is observed in the calculated intermediates, but never by more than 7° and generally around 2–4°. We do not believe that the pyramidalization observed is large enough to cause much differentiation in the size of the orbital lobes on either face of the enolate at C_3. We conclude, in agreement with work of Kamimura et al., that anti addition in Michael reactions of heteronucleophiles results from the endo alkoxy effect.

Thymidylate Synthase. What do these calculations tell us about the catalytic mechanism of TS? On the basis of the calculated transition structures, crystal structures of the binary complex of TS and dUMP,[34] and molecular dynamics simulations of the binary complex,[35] the enzyme locates the catalytic nucleophile (Cys198) in an ideal position to undergo a Michael addition to dUMP. The nucleophilic sulfur of Cys198 is located 3.46 Å away from C_6 of dUMP and has a SC_6C_5 angle of 95.6° in the crystal structure of the binary complex,[34] close to the values in the MP2/6-31+G(d) transition structure for the addition of HS^- to acrolein (**9**/Figure 3). Very little reorganization of the enzyme is required to reach the transition structure. The calculated barriers should be reasonable estimates for TS since the nucleophile is poorly solvated in the binary complex. Cys198 lies at the bottom of the active site, and the binding of dUMP removes most of the water molecules between the bottom of the active site and dUMP.

This study demonstrates that there is little intrinsic difference between oxygen and sulfur nucleophiles with respect to their activation barriers in gas-phase Michael additions. Two separate studies have examined substitution of serine for Cys198. In one study, the mutant TS catalyzed the methylation of dUMP but at a greatly reduced rate while in the other study the mutation resulted in an inactive enzyme.[26] It is widely accepted that the cysteine nucleophile functions much better than a serine nucleophile in TS because of the large pK_a difference for these amino acids. At physiological pH serine is in its neutral state, making it a poor nucleophile. Serine proteases require the catalytic triad to make serine a functional nucleophile. Cysteine's lower pK_a results in the nucleophile being in its anionic form ~10% of the time at physiological pH, assuming its pK_a is unperturbed by its environment. On the basis of pK_as, the formation of a cysteine nucleophile is favored by ~8 kcal/mol over formation of a serine nucleophile. This study reinforces this idea, since there is little intrinsic difference between the nucleophiles in gas-phase Michael additions.

Acknowledgment. P.A.K. is glad to acknowledge research support from the NIH (GM-29072) and the NSF (CHE-94-17458) and we are glad to acknowledge the use of the UCSF Computer Graphics Lab, supported by RR-1081, T. Ferrin, P.I. Some of these calculations were run on the SDSC C90.† Present address: Procept, Inc., Cambridge, MA 02139.

JO951178K

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