Enzyme Mechanisms

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Does Cob(II)alamin Act as a Conductor in Coenzyme B_{12} Dependent Mutases?**

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The origin of the enormous catalytic activity of enzymes containing coenzyme B_{12} as a cofactor continues to be of significant interest in bioinorganic chemistry.[1] During enzymatic catalysis, the Co–C bond of coenzyme B_{12} is cleaved homolytically, which leads to the formation (at least formally) of the 5′-deoxyadenosyl radical and cob(II)alamin.[1,2] The rate of enzymatically accelerated homolytic cleavage of the Co–C bond exceeds the rate observed in aqueous solution by at least 12 orders of magnitude as a consequence of coenzyme interaction with the substrate in the presence of the apoenzyme.[3] The question of how B_{12} enzymes facilitate the homolysis of the Co–C bond and how precisely this initial step is coupled with subsequent H-atom abstraction remains one of the most important and poorly understood aspects of B_{12}-dependent enzymatic catalysis.

Although it is generally accepted that coenzyme B_{12} is only required to initiate the radical reaction and does not participate actively in subsequent steps, an interesting hypothesis was presented recently by Buckel et al.,[4] who proposed that in mutases cob(II)alamin is involved in the stabilization of the product-related highly reactive methylene radical, which is not conjugated to any adjacent group, and that the concept of coenzyme B_{12} as a spectator[5] can only be applied to class II B_{12}-dependent enzymes (eliminases) exemplified by diol dehydratase[6] and glycerol dehydratase.[7] In the light of the results of several experiments, the concept that coenzyme B_{12} acts as a spectator can not be extended to class I B_{12}-dependent enzymes (mutases) exemplified by methylmalonyl CoA mutase[8] and glutamate mutase.[9]

Most noticeably, eliminases are remarkably promiscuous in tolerating structural alterations in the adenosyl moiety, whereas, in sharp contrast, mutases are absolutely specific and do not tolerate any structural modifications. Furthermore, extensive EPR studies and analysis of the intermediate state upon Co–C bond cleavage reveals that the distance between the cob(II)alamin and the substrate radical is much shorter in mutases than in eliminases. Consequently, the interaction between radical centers in mutases is strong relative to that in eliminases. In certain eliminases, coenzyme B_{12} is not needed to initiate the radical reaction, as, for example, in the case of B_{12}-dependent glycerol dehydratase. The same radical reaction can be promoted by a much simpler cofactor, (*S*)-adenosylmethionine (SAM), whereby the 5′-deoxyadenosyl radical is generated by one-electron reduction.[10] Thus, it appears that coenzyme B_{12} is not just a radical generator in mutases; rather cob(II)alamin here functions more like a "conductor" to assist the radical reaction by stabilizing intermediate radicals and lowering transition states in the interconversion of these radicals.[4,11,12]

The purpose of the study described herein was to investigate this fundamental issue by using density functional theory (DFT) and focus on: 1) the structure of the transition state of the concerted Co–C bond homolysis and subsequent H-atom transfer, and 2) the energy stabilization due to the presence of cob(II)alamin. With this objective, we used the model complex Im-[Co^{III}(corrin)]-Rib^+, which contains a corrin ring as well as imidazole and ribose as axial ligands (Figure 1). Previous studies have demonstrated that such structural models describe the electronic properties of cobalamins sufficiently.[13] However, the application of the appropriate level of theory appears to be essential for the correct prediction of the Co–C bond strength.[14] Recent studies have demonstrated that the commonly used B3LYP functional underestimates significantly the strength of the Co–C bond, whereas the nonhybrid BP86 functional provides results that are highly consistent with experiment.[15]

In the present study we made use of these recent developments and used the BP86/6-31G(d) level of theory (see the Methods Section for details) to investigate the initial step of B_{12}-dependent enzymatic catalysis, which involves H-atom abstraction initiated by homolysis of the Co–C bond of coenzyme B_{12}. The optimized Co–C bond length of the

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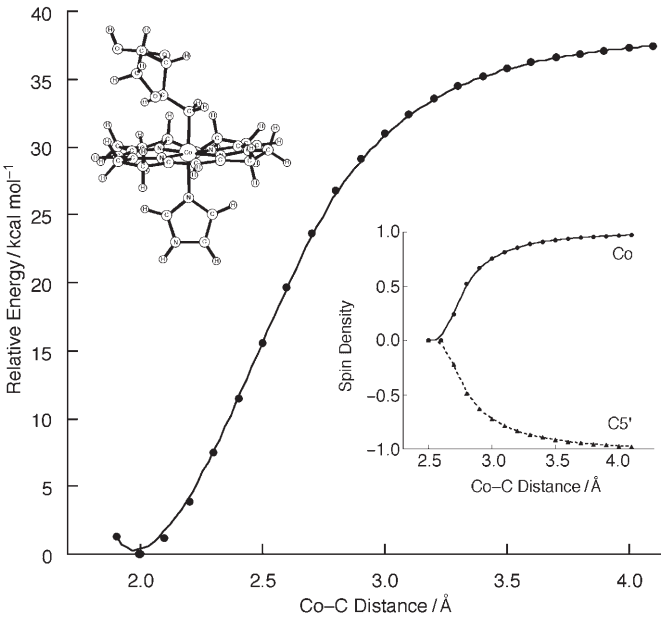

Figure 1. Potential-energy curve that represents the Co–C stretch in the depicted coenzyme-B_{12} model computed at the BP86/6-31G(d) level of theory. The inserted diagram shows the change in the spin density along the Co–C bond elongation.

coenzyme of 1.993 Å is in good agreement with the corresponding distance of 2.033 Å in the crystal structure of adenosylcobalamin (AdoCbl).[16] The Co–C bond dissociation energy (BDE) is predicted to be 37.2 kcal mol^{-1} upon inclusion of the correction for the zero-point vibrational energy (ZPE); this value is in accord with the experimental value of 31 kcal mol^{-1}.[17] These results indicate that the structural model of coenzyme B_{12} is appropriate for the investigation of the initial catalytic step involving hydrogen-atom transfer.

The structure of Im-[Co^{III}(corrin)]-Rib$^+$ in conjunction with the energy curve, which represents the stretching of the Co–C bond (Figure 1), reproduces well the essential features of coenzyme B_{12}. The spin densities on the Co and C5′ atoms were calculated along the Co–C elongation (see inset in Figure 1) and indicate that the diradical character of the coenzyme does not appear until the separation between the cobalt center and the ribose ligand reaches ≈2.7 Å. Full diradical character is developed when the Co–C bond is stretched to more than 3.0 Å. Consequently, the ribose moiety only has the ability to abstract hydrogen from the substrate above this separation.

To explore the realistic energy path associated with the H-atom abstraction, the initial geometry of the transition state (TS) was constructed on the basis of the optimized structure of the cofactor, Im-[Co^{III}(corrin)]-Rib$^+$. A truncated model of the substrate (S) for glutamate mutase, H_3C–C**H**H–CHO was placed near the C5′ atom for direct H-atom abstraction (of the H atom in bold face), for which an accurate X-ray structure analysis exists.[9b] This simple substrate model was used in full quantum-mechanical calculations at the BP86/6-31G(d) level of theory to search for the transition state. These types of models have been used in previous theoretical studies on radical rearrangements in conjunction with B_{12}-dependent carbon-skeleton mutases.[18]

To initiate the transition-state (TS) search a series of constrained optimizations were carried out, and in the final stage full optimization was performed with default optimization criteria. The validity of the calculated TS was confirmed by frequency calculations. This TS has only one imaginary frequency (905*i* cm^{-1}), which corresponds to the hydrogen-atom motion associated with C–H bond cleavage and subsequent H-atom transfer to C5′ (Figure 2). There is no

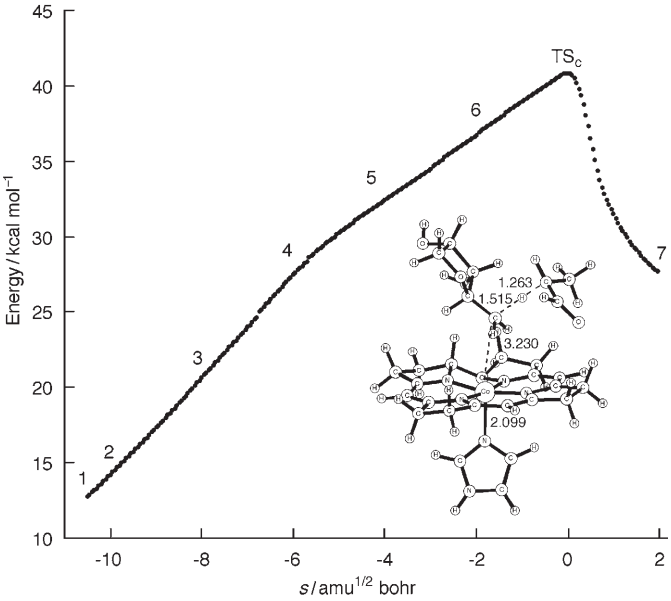

Figure 2. The transition-state structure of a homolytic Co–C bond cleavage concerted with a subsequent H-atom abstraction is shown along with the energy profile derived from IRC calculations where *s* is the distance along the IRC.

motion along the Co–C coordinate in this imaginary frequency mode that can be attributed to the kinematic effect associated with the Co/H mass ratio. Intrinsic-reaction-coordinate (IRC) calculations were carried out to verify the identity of the located TS and to demonstrate that this TS is connected with the Co–C bond homolysis and subsequent H-atom abstraction (see Figure 2 and Table 1 for a summary). The IRC calculations reveal that the product (labeled 7) is the radical pair composed of cob(II)alamin and S$^{\bullet}$, which results from homolysis. On the reactant side, the IRC path smoothly connects the TS with the reactant complex (RC_c) and shows the absence of any intermediate that would be indicative of a stepwise mechanism. Thus, the IRC energy profile confirms that the located TS is indeed associated with a concerted mechanism.

The structure of the TS gives further insight into the mechanism associated with the initial H-atom abstraction by coenzyme B_{12}. The long Co–C bond of 3.230 Å is the most prominent structural feature of the TS; its length is similar to that of 3.2 Å present in the crystal structure of the 2′-*endo* conformation (population A) of glutamate mutase.[9b] It is also comparable to the distance of 2.99 Å between the cobalt

Table 1: Selected distances and spin densities along the IRC pathway (see Figure 2).

	1	2	3	4	5	6	TS	7
					Distance [Å]			
Co-C5′	2.247	2.283	2.451	2.674	2.841	3.054	3.230	3.221
C5′···H	3.058	3.031	2.895	2.683	2.464	2.020	1.515	1.107
H-C_{sub}	1.113	1.113	1.113	1.112	1.112	1.121	1.263	1.866
					Spin density			
Co	0.0	0.0	0.0	0.4	0.7	0.9	0.9	1.0
C5′	0.0	0.0	0.0	−0.4	−0.7	−0.8	−0.6	−0.1
corrin	0.0	0.0	0.0	0.0	−0.1	−0.1	−0.1	−0.1
C_{sub}	0.0	0.0	0.0	0.0	0.0	−0.1	−0.3	−0.7

center and the C5′ atom of the adenosine ribose moiety in a synthetic analogue of coenzyme B_{12} in which this distance was artificially enhanced with an additional CH_2 unit.[19] The length of the H–C_{sub} bond is only stretched modestly from 1.113 Å to 1.263 Å, and the C5′···H distance of 1.515 Å is still relatively long. Thus, the TS occurs late along the Co–C coordinate, but early with respect to the H-atom transfer. Analysis of the changes in the electronic structure along the reaction coordinate (Table 1, points 1–7) indicates that initial biradical character appears when the separation between the cobalt atom and the ribose ligand reaches approximately 2.7 Å (point 4), and this biradical character of the free cofactor is not altered by the presence of substrate (Figure 1). The Co–C bond has to be stretched further to 3.2 Å, and the C5′ atom has to gain additional spin density (more than 0.9) before the ribose moiety has the ability to abstract a hydrogen atom. After this point the H-atom transfer occurs rapidly from the substrate to ribose (point 7).

Although the located TS is described as having no motion along the Co–C coordinate, the reaction initiated by homolytic cleavage of the Co–C bond and subsequent H-atom transfer is concerted (Figure 2). The concerted nature of the reaction further implies that the transition state, in which the H-atom is partially transferred between the substrate and the ribose ligand, should be stabilized by the presence of cob(II)alamin, consistent with the recent proposal of Buckel et al.[4] To estimate the magnitude of such a stabilization, both stepwise and concerted pathways were analyzed and compared (Figure 3). To make a quantitative prediction, the reference energy was defined with respect to Im-[Co^{III}-(corrin)]-Rib^+ and the substrate at infinite separation (B_{12}+S). The stepwise reaction is initiated by homolysis of the Co–C bond, predicted to occur at 37.2 kcal mol^{-1}. This step leads to cob(II)alamin and a ribose radical (Rib•), which later abstracts a hydrogen atom from the substrate. The reaction complex (RC_s) formed between Rib• and S is only stabilized by 0.5 kcal mol^{-1} and is no longer affected by the presence of cob(II)alamin. The resulting transition state (TS_s), in which the hydrogen atom is partially transferred from the substrate to the ribose radical is predicted to lie at 40 kcal mol^{-1}. At this stage the cob(II)alamin appears only as a spectator, and its energy has been added to the energy of TS_s. The same analysis for the concerted pathway results in a calculated binding energy for RC_c of 3.0 kcal mol^{-1}. The difference between the binding energy of RC_s and that of RC_c is derived from the larger interaction energy between the cofactor and the substrate in RC_c. Consequently, the resulting transition state (TS_c) is now predicted to lie at 33.0 kcal mol^{-1} upon inclusion of the ZPE correction, which for the concerted pathway is predicted to be 4.0 kcal mol^{-1}. Thus, a comparison of these two transition states reveals that H-atom abstraction by the concerted pathway is stabilized by 7.0 kcal mol^{-1} because of the

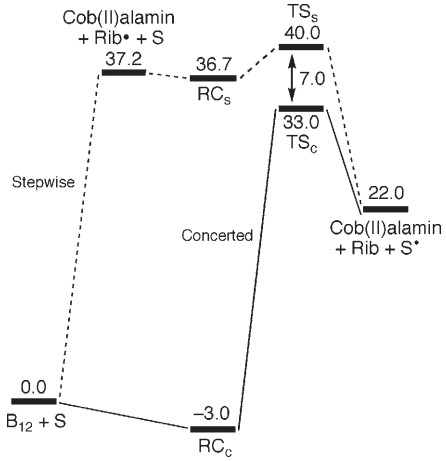

Figure 3. Comparison of concerted and stepwise energetic pathways. All energies were corrected with respect to the ZPE.

presence of cob(II)alamin. This stabilization of TS_c reflects the ability of the ribose moiety to abstract a hydrogen atom and correlates with the amount of spin density, ρ(C5′), built up on the C5′ center. The spin polarization takes place after the separation between the cobalt atom and the C5′ center becomes greater than 2.7 Å; it increases monotonically and becomes close to one when the distance is greater than 4.2 Å (Figure 1). A distance of 4.2 Å would essentially correspond to complete cleavage of the Co–C bond. Consequently, TS_s can be viewed as the transition state along a concerted pathway, in which an elongation of the Co–C bond is at least 4.2 Å and the unit spin density is built up on the carbon center (Figure 3). However, our calculations show that at a distance of 3.2 Å, when $\rho(C5') \approx 0.9$ (Table 1), the C5′ center already has enough strength to abstract a hydrogen atom. Thus, according to the energy curve in Figure 1, the stabilization of TS_c is primarily derived from the energy difference for full and partial cleavage at which H-atom abstraction takes place. Since spin density can be tuned with the change in the Co–C distance, this argument further implies that the stabilization of TS_c would depend on the type of hydrogen atom that is abstracted; that is, the stabilization would be greater for hydrogen atoms at secondary carbon centers than for those at primary centers.

In conclusion, the present study constitutes the first computational analysis of the proposal that cob(II)alamin

stabilizes radical intermediates in coenzyme B_{12} dependent mutases.[4] It was shown that the initial cleavage of the Co–C bond and subsequent H-atom abstraction may occur in a concerted fashion, and that these two reactions can together contribute to the energetics of enzymatic activation by 7 kcal mol^{-1}. This amount of energy, by which the transition state is stabilized, is in agreement with the value of 8 kcal mol^{-1} discussed recently by Brown.[12] It is emphasized that the current analysis has been carried out for the substrate and isolated cofactor without explicit inclusion of the protein environment. Although recent QM/MM studies[20,21] did not find any lowering of the energy of H-atom transfer between the substrate and the adenosyl radical because of the presence of cob(II)alamin, we believe that this issue needs to be investigated further in light of the present analysis.

Methods Section

All DFT calculations were carried with the Becke–Perdew[22] (BP86) functional. The initial structural analysis was performed by using Jaguar[23] and the LACVP(Co)/6-31G*(rest) basis set. The final geometry optimization and frequency calculations were carried out with the BP86 functional and the 6-31G(d)(5d) basis set as implemented in the Gaussian[24] suite of programs for electronic-structure calculations. All transition states were characterized by the convergence of their Hessians followed by frequency calculations. The IRC calculations were carried out to verify the identity of the located TS. The data generated by the study are collected in the Supporting Information.

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[1] a) L. G. Marzilli in *Bioinorganic Catalysis* (Ed.: J. Reedijk), Marcel Dekker, New York, **1993**, pp. 227–259; b) *Vitamin B_{12} and B_{12} Proteins* (Eds.: B. Kräutler, D. Arigoni, B. T. Golding), Wiley-VCH, New York, **1998** (Lectures Presented at the 4th European Symposium on Vitamin B_{12} and B_{12} Proteins); c) R. Banerjee, *Chemistry and Biochemistry of B_{12}*, Wiley, New York, **1999**; d) T. Toraya, *Chem. Rev.* **2003**, *103*, 2095–2127; e) T. Toraya, *Cell. Mol. Life Sci.* **2000**, *57*, 106–127; f) R. Banerjee, *Chem. Rev.* **2003**, *103*, 2083–2094.

[2] J. Halpern, *Science* **1985**, *227*, 869–875.

[3] a) B. P. Hay, R. G. Finke, *J. Am. Chem. Soc.* **1987**, *109*, 8012–8018; b) R. G. Finke in *Vitamin B_{12} and B_{12} Proteins* (Eds.: B. Kräutler, D. Arigoni, B. T. Golding), Wiley-VCH, Weinheim, **1998**, chap. 25.

[4] W. Buckel, B. T. Golding, C. Kratky, *Chem. Eur. J.* **2006**, *12*, 352–362.

[5] B. T. Golding, L. Radom, *J. Am. Chem. Soc.* **1976**, *98*, 6331–6338.

[6] a) N. Shibata, J. Masuda, T. Tobimatsu, T. Toraya, K. Suto, Y. Morimoto, N. Yasuoka, *Structure* **1999**, *7*, 997–1008; b) N. Shibata, J. Masuda, T. Tobimatsu, T. Toraya, K. Suto, Y. Morimoto, N. Yasuoka, *Structure* **1999**, *7*, 997–1008; c) N. Shibata, J. Masuda, Y. Morimoto, N. Yasuoka, T. Toraya, *Biochemistry* **2002**, *41*, 12607–12617; d) N. Shibata, Y. Nakanishi, M. Fukuoka, M. Yamanishi, N. Yasuoka, T. Toraya, *J. Biol. Chem.* **2003**, *278*, 22717–22725.

[7] a) M. Yamanishi, M. Yunoki, T. Tobimatsu, H. Sato, J. Matsui, A. Dokiya, Y. Iuchi, K. Oe, K. Suto, N. Shibata, Y. Morimoto, N. Yasuoka, T. Toraya, *Eur. J. Biochem.* **2002**, *269*, 4484–4494; b) D.-I. Liao, G. Dotson, I. Turner, Jr, L. Reiss, M. Emptage, *J. Inorg. Biochem.* **2003**, *93*, 84–91.

[8] a) F. Mancia, N. H. Keep, A. Nakagawa, P. F. Leadlay, S. McSweeney, B. Rasmussen, P. Bosecke, O. Diat, P. R. Evans, *Structure* **1996**, *4*, 339–350; b) F. Mancia, P. R. Evans, *Structure* **1998**, *6*, 711–720; c) F. Mancia, G. A. Smith, P. R. Evans, *Biochemistry* **1999**, *38*, 7999–8005.

[9] a) R. Reitzer, K. Gruber, G. Jogl, U. G. Wagner, H. Bothe, W. Buckel, C. Kratky, *Structure* **1999**, *7*, 891–902; b) K. Gruber, R. Reitzer, C. Kratky, *Angew. Chem.* **2001**, *113*, 3481–3484; *Angew. Chem. Int. Ed.* **2001**, *40*, 3377–3380.

[10] a) C. Raymond, P. Sarcabal, I. Meynial-Salles, C. Croux, P. Soucaille, *Proc. Natl. Acad. Sci. USA* **2003**, *100*, 5010–5015; b) J. R. O'Brien, C. Raynaud, C. Croux, L. Girbal, P. Soucaille, W. N. Lanzilotta, *Biochemistry* **2004**, *43*, 4635–4645.

[11] J. M. Pratt in *Chemistry and Biochemistry of B_{12}* (Ed.: R. Banerjee), Wiley, New York, **1999**, p. 113.

[12] K. L. Brown, *Chem. Rev.* **2005**, *105*, 2075–2149.

[13] a) K. P. Jensen, U. Ryde, *J. Mol. Struct. (Theochem)* **2002**, *585*, 239–255; b) P. M. Kozlowski, M. Z. Zgierski, *J. Phys. Chem. B* **2004**, *108*, 14163–14170.

[14] K. P. Jensen, U. Ryde, *J. Phys. Chem. A* **2003**, *107*, 7539–7545.

[15] J. Kuta, S. Patchkovskii, M. Z. Zgierski, P. M. Kozlowski, *J. Comput. Chem.* **2006**, *27*, 1429–1437.

[16] L. Ouyang, P. Rulis, W. Y. Ching, G. Nardin, L. Randaccio, *Inorg. Chem.* **2004**, *43*, 1235–1241.

[17] a) B. P. Hay, R. G. Finke, *J. Am. Chem. Soc.* **1986**, *108*, 4820–4829; b) R. G. Finke, B. P. Hay, *Polyhedron* **1988**, *7*, 1469–1481.

[18] S. D. Wetmore, D. M. Smith, B. T. Golding, L. Radom, *J. Am. Chem. Soc.* **2001**, *123*, 7963–7972.

[19] a) S. Gschösser, R. B. Hannak, R. Konrat, K. Gruber, C. Mikl, C. Kratky, B. Kräutler, *Chem. Eur. J.* **2005**, *11*, 81–93; b) M. Fukuoka, Y. Nakanishi, R. B. Hannak, B. Kräutler, T. Toraya, *FEBS J.* **2005**, *272*, 4787–4796.

[20] K. P. Jensen, U. Ryde, *J. Am. Chem. Soc.* **2005**, *127*, 9117–9128.

[21] R. A. Kwiecien, I. V. Khavrutskii, D. G. Musaev, K. Morokuma, R. Banerjee, P. Paneth, *J. Am. Chem. Soc.* **2006**, *128*, 1287–1292.

[22] a) A. D. Becke, *J. Chem. Phys.* **1986**, *84*, 4524–4529; b) J. P. Perdew, *Phys. Rev. B* **1986**, *33*, 8822–8824.

[23] Jaguar 5.0, L. L. C. Schrödinger, Portland, OR, 1991–2003.

[24] Gaussian 03 (Revision A.1), M. J. Frisch, G. W. Trucks, H. B. Schlegel, G. E. Scuseria, M. A. Robb, J. R. Cheeseman, J. A. Montgomery, Jr., T. Vreven, K. N. Kudin, J. C. Burant, J. M. Millam, S. S. Iyengar, J. Tomasi, V. Barone, B. Mennucci, M. Cossi, G. Scalmani, N. Rega, G. A. Petersson, H. Nakatsuji, M. Hada, M. Ehara, K. Toyota, R. Fukuda, J. Hasegawa, M. Ishida, T. Nakajima, Y. Honda, O. Kitao, H. Nakai, M. Klene, X. Li, J. E. Knox, H. P. Hratchian, J. B. Cross, C. Adamo, J. Jaramillo, R. Gomperts, R. E. Stratmann, O. Yazyev, A. J. Austin, R. Cammi, C. Pomelli, J. W. Ochterski, P. Y. Ayala, K. Morokuma, G. A. Voth, P. Salvador, J. J. Dannenberg, V. G. Zakrzewski, S. Dapprich, A. D. Daniels, M. C. Strain, O. Farkas, D. K. Malick, A. D. Rabuck, K. Raghavachari, J. B. Foresman, J. V. Ortiz, Q. Cui, A. G. Baboul, S. Clifford, J. Cioslowski, B. B. Stefanov, G. Liu, A. Liashenko, P. Piskorz, I. Komaromi, R. L. Martin, D. J. Fox, T. Keith, M. A. Al-Laham, C. Y. Peng, A. Nanayakkara, M. Challacombe, P. M. W. Gill, B. Johnson, W. Chen, M. W. Wong, C. Gonzalez, J. A. Pople, Gaussian, Inc., Pittsburgh, PA, **2003**.

