Conformational Analyses of Thiamin-Related Compounds. A Stereochemical Model for Thiamin Catalysis

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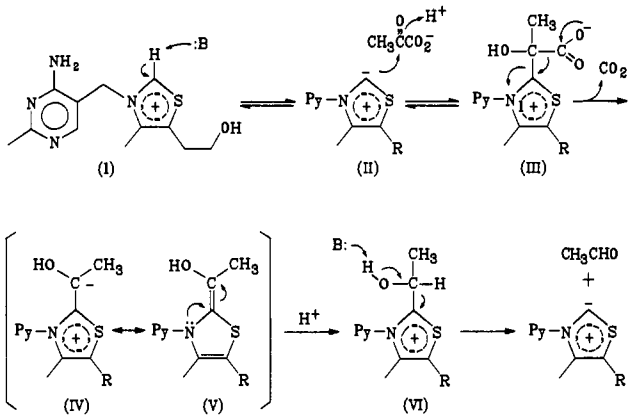
Abstract: Conformational analyses of some thiamin-related compounds have been performed in order to find the relationship between their conformational and biochemical properties. Relaxed 2-D potential energy maps of free thiamin, its antagonists, and C(2) adducts were obtained using the molecular mechanics (MM) method. The antagonists include 4′-deaminothiamin, oxythiamin, pyrithiamin, thiamin thiazolone, thiamin thiothiazolone, 6′-methyl-4*H*-thiamin and 6′-methylthiamin, and the C(2) adducts include 2-(α-hydroxyethyl)thiamin (HET), 2-(α-lactyl)thiamin (LT), 6′-methyl-4*H*-lactylthiamin and 6′-methyllactylthiamin. All of the local minima conformers were also identified for the active intermediates LT and HET. In numerous crystal structures, free thiamin assumes mostly the F form and less frequently the S form. The C(2) adducts assume only the S form. However, neither thiamin nor its active intermediates are found in the V form in crystal, but the cofactor assumes the V form in the active site of the protein. The MM map of free thiamin shows that the F form is truly the global minimum, while the S form occupies another minimum slightly higher in energy and lower in existence probability than the F form. The V form is also a local minimum but with very low existence probability. In addition, the availability of only one 4′-amino H atom for the intermolecular hydrogen bond makes it very unlikely that the thiamin molecule assumes the V form in solution and thereby in crystals. For various antagonists, either the V form is the global minimum or its existence probability is higher than that of thiamin. The V form instead of the crystalline S form is the global minimum conformer for both LT and HET. However, the V form of the C(2) adducts also would not be observed in the crystalline state due to the conformational characteristics of free thiamin; their V forms accordingly may be unique conformers available only inside the protein but not in solution. Based on the results of MM calculations and the crystal structures of holoenzymes, it is proposed that the active conformers of both intermediates are V forms with an intramolecular N–H···O(hydroxyl) hydrogen bond. A putative stereochemical model for thiamin catalysis is presented in which N–H···O hydrogen bonds contribute to the acceleration of the enzymic reaction by lowering the energies of the various species occurring along the reaction path. The principles of least motion and maximum orbital overlap which were originally applied to the decarboxylation reaction for the intermediates in the S form still hold for the V form.

Introduction

Thiamin (vitamin B_1; I), in the form of the diphosphate ester (TDP), is a coenzyme in a number of enzyme systems that catalyze decarboxylation of α-keto acids and the transfer of aldehyde or acyl groups.[1] Since Breslow[2] clarified the essential features of thiamin catalysis (Scheme I) 35 years ago, many details have been clarified from studies of both model and enzyme systems, but some important questions remain to be answered. One of the controversial points, that has been clarified very recently, was the active conformation of thiamin, which in turn is directly related to the role of the essential 4′-amino group in the enzymic catalysis. From extensive biochemical studies employing various analogues of thiamin, Schellenberger concluded that the 4′-amino group actively participates in the catalytic reaction acting as an intramolecular acid and base alternatively and thus thiamin and its C(2) adducts should be in the V form in which the 4′-amino group is close to the C(2) active center.[3] Sable et al. also proposed the stable V form of free thiamin from NMR studies on the model compounds.[4] However, the V form has never been found

Scheme I



in any of the crystal structures of free thiamin or of its C(2) adducts, making the active conformation of thiamin a controversial subject. Very recently, the crystal structures of transketolase,[5] pyruvate oxidase,[6] and pyruvate decarboxylase (PDC)[7] that require TDP as a cofactor have been reported at 2.5, 2.1, and 2.8 Å resolutions, respectively. They vividly show that *the active conformation of thiamin is the V form.* A protein structure

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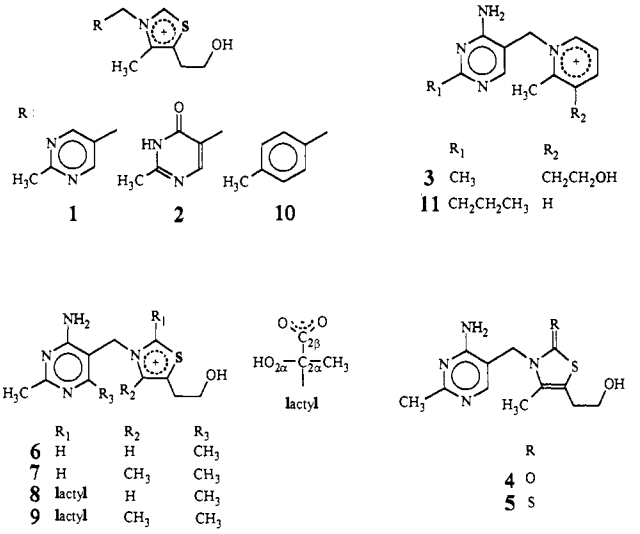
containing an active intermediate such as 2-(α-lactyl)thiamin [lactylthiamin, LT; III] or 2-(α-hydroxyethyl)thiamin (HET; VI) has not been determined yet. However, the protein structures strongly suggest that the 4′-amino group somehow plays a direct role through some kinds of intramolecular interactions during the catalysis as suggested by Schellenberger.

In numerous crystal structures, free thiamin assumes mostly the F conformation and with minor exceptions the S conformation despite the apparent rotational degree of freedom about the bridging methylene joining the two aromatic rings.[8] Furthermore, all C(2)-substituted thiamins assume only the S conformation although the conformations of the substituents are variable. They include HET,[9] 2-(α-hydroxybenzyl)thiamin[10] and its Hg complex,[11] 2-(α-hydroxybenzyl)oxythiamin,[12] and phosphalactylthiamin (PLT).[13] In both the F and S forms, the 4′-amino group is far from the C(2) active center and thus cannot be directly involved in intramolecular acid–base catalysis if one of these is the active form. In the crystal structures of thiamin-related compounds, the V conformation has been observed only in three cases, namely, oxythiamin,[14] thiamin thiazolone (TT),[15] and 4′-deaminothiamin analogues[16] all of which are antagonists of thiamin. The crystal structure of TT which contains an intramolecular N–H···O hydrogen bond in the V form has remained as the only example that remotely resembles the Schellenberger's active V model. However, thiamin thiothiazolone (TTT),[17] a structural and functional congener of TT, assumes the S conformation, exemplifying a notion[15] that the crystal conformation of a thiamin compound is a result of an intricate balance of intramolecular interactions. An important question remaining to be answered is why the active V form has virtually never been found for free thiamin or for the active intermediates in the crystalline state.

Despite prolonged controversy over the conformational properties of thiamin-related compounds, extensive theoretical studies have not been reported yet. Conformational energy maps of thiamin and HET were obtained in terms of torsion angles of ϕ_T and ϕ_P but without geometry optimization.[18] In particular, the calculation for HET was performed for a fixed conformer of the C(2)-substituent so that it does not represent the available conformational surface adequately.[18b] No conformational studies have been done on the antagonists of thiamin although their biochemical behavior provided the basis of some important mechanistic proposals. In this work we have calculated relaxed 2-D conformational potential energy maps for free thiamin and its biologically important antagonists and C(2) adducts using molecular mechanics (MM). The antagonists include 4′-deaminothiamin (**1**), oxythiamin (**2**), pyrithiamin (**3**), TT (**4**), TTT (**5**), 6′-methyl-4*H*-thiamin (**6**), and 6′-methylthiamin (**7**). The C(2) adducts include HET, LT, 6′-methyl-4*H*-lactylthiamin (**8**), and 6′-methyllactylthiamin (**9**). All of the local minima conformers of HET and LT were also identified. Although the diphosphate esters of the above compounds are biologically active, we have disregarded the diphosphate ester moiety in the calculation since it does not affect the conformational properties of the thiamin moiety and the C(2)-substituent. In the present study we find the reason why the V form has not been observed in crystal structures of free thiamin and C(2) adducts. In addition we correlate the conformational and biochemical properties of the various analogues. We also propose a possible stereochemical course of thiamin catalysis.

Chart I



Computational Methods

In order to obtain the fully minimized conformational energy maps, we used an in-house MM program which has several salient features.[19] The various potential energy functions and the parameters were taken from Allinger's MM2(85)[20] with a couple of modifications. We included the charge/charge, instead of the dipole/dipole, interaction energy and explicit hydrogen-bonding interaction energy terms. The functional form of the charge interaction energy is $q_iq_j/\epsilon r_{ij}$. Partial atomic charges were derived by application of the Mulliken population analysis routine in AM1.[21] The distance-dependent dielectric constant of $\epsilon = 4r_{ij}$ (r_{ij} in Å) was used in this study. The hydrogen-bonding interaction energies were calculated with the Buckingham function which was modulated with angle factor terms taken from YETI.[22] ϵ_0 (well depth) and r_0 (equilibrium distance) parameters for hydrogen-bonding energy were set at −3.483 kcal/mol and 1.877 Å for N–H···O and −2.50 kcal/mol and 2.30 Å for N–H···S, respectively.[23] A switching function was not used because of the mathematical complexity in the derivation of the derivatives. Instead, the H···O (or S) interaction was treated as a normal van der Waals interaction, when the distance between the H and the acceptor atoms exceeded $1.5r_0$. X-ray bond distances and angles involving the non-hydrogen atoms were used as l_0 of the bond stretching and θ_0 of the angle bending terms. Those involving the hydrogen atoms were the normalized values. In our program, energy minimization is done using the conjugate gradient minimizer in the internal coordinate system rather than the

(8) The conformation of the thiamin molecule is best expressed in terms of the two torsion angles, ϕ_T and ϕ_P, about the bonds from the methylene bridge carbon to the thiazolium and pyrimidine rings, respectively. The torsion angles ϕ_T = C(5′)–C(3,5′)–N(3)–C(2) and ϕ_P = N(3)–C(3,5′)–C(5′)–C(4′). The F, S, and V conformations have been specified by ($\phi_T \approx 0°$, $\phi_P \approx \pm 90°$), ($\phi_T \approx \pm 100°$, $\phi_P \approx \pm 150°$), and ($\phi_T \approx \pm 90°$, $\phi_P \approx \mp 90°$), respectively. For more details, see: footnote 13 in ref 10. Their locations in the (ϕ_T, ϕ_P) map are specified in Figure 3a.

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orthogonal system. It is possible to fix any structural variable during minimization in this scheme, and thus the problems that are associated with the dihedral driver scheme originally implemented in MM2 can be avoided.[24]

The MM calculations of the conformational potential energy maps of thiamin and its antagonists were performed on 5° grids of (ϕ_T, ϕ_P) with full geometry optimization (except for the fixed torsion angles of ϕ_T and ϕ_P). Contouring was done with an in-house program. The 2D-energy maps of C(2)-substituted thiamins and analogues were obtained by the newly developed iterative four-way scanning method incorporated in our MM program. Individual local minima conformers of HET and LT were found by application of a systematic grid search method in which a unique minimization scheme is employed in the search process for computational efficiency. First, each structure that is generated varying the rotatable bonds is minimized with respect only to the torsion angles associated with the rotatable bonds, and the local minima unique in terms of the torsion angles are saved. Then, the structures are subject to further minimization of the bond angles and the torsion angles associated with the rotatable bonds and screened again. Finally, the local minima are identified from a comparison of the fully minimized structures. A conformer is defined as unique when its torsion angles show an rms difference larger than a certain value (15° in the present study) from those of the other conformers. This procedure gives better results than the grid search method with fixed geometry while keeping the computation feasible even on the fast PC. We could even distinguish the conformers that differed only in the orientation of the H atom of the hydroxyl group. All calculations were performed on either a PC 486 or an IBM RS/6000 computer at the Seoul National University Computer Center.

Analyses of Crystal Structures of Thiamin

Conformation. In order to investigate the structural and conformational properties of thiamin in detail, we first made detailed analyses of the crystallographic data. Previously, Shin et al. had tabulated the occurrence of 17 thiamin-related compounds in various conformations.[25] Since then, the number of the crystal structures has almost quadrupled (62 crystal structures in 57 independent determinations), and several more cases of free thiamin occurring in the S form have been reported. It seemed timely to make an updated list (Table I) which could be informative for discussing the conformational properties of thiamin-related compounds. The compounds have been classified in terms of the chemical variations on various sites of thiamin as well as their conformations.

For free thiamin, the F form is overwhelmingly preferable to the S form (43 *versus* 8 cases). The average ϕ_T and ϕ_P angles for the F form are $-4 \pm 7°$ and $-83 \pm 6°$, respectively. The first time that free thiamin was found in the S conformation, it was suggested that the F form is an artifact of crystal packing forces.[26] Since then, eight cases of the S form have been reported. In most of them the thiamin molecule is associated with a polyhalogenicmetal anion except for the picrate complex. Actually, however, only four of the cases are unique since two salts ($CdCl_4$ and $CoCl_4$) and four metal (Zn, Co, Cd) complexes represent two isomorphous structures, as can be seen in the very similar torsion angles and the same space groups. The average ϕ_T and ϕ_P angles for this conformer of thiamin are $-112 \pm 1°$ and $-134 \pm 4°$, respectively. These angles are significantly different from those of the S form of C(2)-substituted thiamin in which the average ϕ_T and ϕ_P angles are $-95 \pm 7°$ and $175 \pm 7°$, respectively. Since ϕ_P differs by $\sim 50°$ and since there are other structural differences, notably in the bond angles, it seems better to distinguish these two conformations. We tentatively designated the conformation of free thiamin as S′. Thiamin·$HgCl_4$·H_2O[27] and HET[9] which assume the S and S′ forms, respectively, are exceptional cases. In the S′ form, H(6′) is directed toward C(2), while it is right on top of N(3) in the S form. The crystallographic data suggest that chemical perturbations at the C(2) or the C(4′) position affect the conformation of free thiamin but perturbations at N(1′) or O(5γ) do not. In fact, the torsion angles $\phi_{5\alpha}$ [S(1)–C(5)–C(5α)–C(5β)] and $\phi_{5\beta}$ [C(5)–C(5α)–C(5β)–O(5γ)] involving the 5-hydroxyethyl side chain vary widely among the various crystal structures. Therefore, we can assume that the major conformational properties of TDP are identical to those of thiamin as far as the torsion angles ϕ_T and ϕ_P are concerned. As is evident in Table I, thiamin can be crystallized with a variety of counter ions or complexing agents and in different space groups, indicating that the crystal packing modes around the thiamin molecule are quite different. Therefore, these data are statistically convincing evidence that the preferred conformations (major F and minor S) of free thiamin represent an intrinsic conformational property of the molecule rather than an artifact of crystal packing forces. MM studies presented below prove that this interpretation is correct. They also show that the most stable conformation of C(2)-substituted thiamins is the V form and that their crystalline S conformations are the combined result of the conformational property of thiamin in solution, crystallization processes, and crystal packing forces, as shall be discussed later.

Molecular Dimensions. It has been observed that the molecular dimensions of the thiamin molecule show some systematic variations that are associated with the chemical and conformational changes. Cramer et al. have observed that bond lengths and endocyclic bond angles of the pyrimidine ring moiety are subject to the protonation state of the ring.[28] They also observed that the two exocyclic angles around N(3) are sensitive to the molecular conformation, that is, C(2)–N(3)–C(35′) is 1–3° smaller than C(4)–N(3)–C(35′) for free thiamin in the S conformation, while the opposite trend is true for the F form.[28] In addition, they concluded that for the angles around C(5′) there are no obvious systematic variations depending on conformation. However, we have found that there are indeed systematic variations in the exocyclic angles around C(5′) depending only on the conformation, that is, the average value of C(4′)–C(5′)–C(35′) is smaller by 3.5° than that of C(6′)–C(5′)–C(35′) for C(2)-substituted thiamin in the S form but larger by 2.7° for free thiamin in both F and S′ form. The average molecular dimensions of thiamin which show significant differences depending on the protonation state and conformational classes are shown in Figure 1.

Atomic Charges of Thiamin

AM1 calculations for all compounds studied were performed mainly for obtaining the atomic charges for use in the MM energy calculations. The net atomic charges for protonated and unprotonated thiamins are shown in Figure 2. It has been found that the atomic charges in the thiazolium ring show different values depending upon the methods of calculation.[18a,b,29] Although the thiazolium ring is commonly drawn with a formal positive charge on N(3), the AM1 results show that S(1) carries nearly unit positive charge, while C(2) and C(4) carry considerable

(23) In general, the parameters for N–H···O are rather well-defined and our present MM calculation with the above parameters gave the results consistent with the crystal structure and the AM1 results. However, parameters for the N–H···S hydrogen bond have not been studied in detail. The ϵ_0 and r_0 values in YETI are −1.455 kcal/mol and 2.667 Å, respectively, but those are for the thiol or thioether S atom and may not be suitable for TTT with the thioketo S atom. Therefore, we tentatively used the values of $\epsilon_0 = -2.50$ kcal/mol and $r_0 = 2.30$ Å to provide the most favorable condition for the formation of an intramolecular hydrogen bond. These values are based on the shortest H···S distance of 2.27 Å observed in the crystal structures of thio derivatives of the nucleic acid components which have the intermolecular N–H···S hydrogen bond (Saenger, V. W.; Suck, D. *Acta Crystallogr., Sect. B* **1971**, *27*, 1178–1186) and the hydrogen-bonding energy of −2.50 kcal/mol predicted for the $CHONH_2$–H_2CS system (Kollman, P.; McKelvey, J.; Johansson, A.; Rothenberg, S. *J. Am. Chem. Soc.* **1975**, *97*, 955–965).

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Table I. Torsion Angles (deg) and Space Groups in the Crystal Structures of Thiamin and Its Analogues[a]

form	ϕ_T	ϕ_P	sp gp	form	ϕ_T	ϕ_P	sp gp
F Form							
(1) Free Thiamin Salt							
(T)Cl	−2.9	−85.3	$P2_1/a$	(T)PF_6·H_2O	−0.5	−83.2	$P2_1/n$
(T)Cl·H_2O	−2.6	−76.8	$P2_1/c$	(HT)$2PF_6$·$4H_2O$	−8.9	−87.2	$P2_1/a$
(HT)Cl_2·$^1/_2H_2O$	−6.4	−76.0	$C2/c$	(T)thiocyanate	5.8	−86.6	$C2/c$
(HT)Cl_2·H_2O	−9.0	−76.1	$P2_1/c$	(T)thiocyanate·H_2O (A)	−4.6	−87.7	$P\bar{1}$
(HT)Br_2·$^1/_2H_2O$	−2.3	−77.1	$C2/c$	(B)	−7.3	−90.9	
(T)Br·$1.5H_2O$ (A)	−6.8	−76.6	$P2_1/a$	(HT)Cl·$^1/_2(SO_4)$·H_2O	−1.3	−76.2	$C2/c$
(B)	−4.4	−82.0		(T)(picrolonate)·$2H_2O$	−5.9	−82.4	$P\bar{1}$
(HT)I_2	5.9	−81.0	$P2_1/c$	(HT)(naphthalene-1,5-disulfonate)·H_2O	−20.9	−79.0	$P2_12_12_1$
(T)(NO_3)	−5.9	−83.1	$P2_1/n$	(HT)$^1/_2[Mg(H_2O)_6]Cl_3$·$2H_2O$	−2.0	−77.4	$P\bar{1}$
(HT)$(NO_3)_2$	−13.9	−77.5	$P\bar{1}$	(HT)$(CuCl_4)$	−14.1	−82.6	$P2_1/c$
(T)(ClO_4)·H_2O	−2.3	−83.2	$P2_1/n$	(HT)$(PtCl_4)$	−9.0	−76.7	$P\bar{1}$
(HT)·$2ClO_4$	10.4	−81.5	$P2_1/n$	$(HT)_2(PtCl_4)Cl_2$·$2H_2O$	0.2	−85.8	$P\bar{1}$
(T)BF_4·H_2O	−3.0	−82.4	$P2_1/n$	(HT)$^1/_2(UO_2Cl_4)$Cl	5.4	−83.7	$P2_1/n$
(2) Free Thiamin Substituted at or Coordinated to N(1′)							
(1′-methylT)I_2	−3.7	−83.7	$P2_1$	$Zn(T)(SCN)_3$	−10.2	−82.8	$P2_1/a$
Cu(T)Cl_2	10.1	−83.7	$P\bar{1}$	[Mn(T)Cl_2·H_2O]·(HT)Cl_2·H_2O	−10.0	−85.6	$P\bar{1}$
Cu(T)Br_2	12.0	−82.1	$P\bar{1}$		−3.6	−79.6	
Pt(T)Cl_3·H_2O	−5.3	−70.0	$P\bar{1}$	[Rh(T-PO_3H)(acetato)$_2$]·$0.75H_2O$	−3.3	−80.7	$P\bar{1}$
(3) Free Thiamin Substituted at O(5γ)							
(HT-PO_3H)(H_2PO_4)·$3H_2O$	−6.6	−85.4	$P\bar{1}$	(HT-P_2O_6H)·$4.5H_2O$	−8.5	−92.4	$P\bar{1}$
(HT-PO_3)PF_6·$2H_2O$	−9.0	−78.8	$P\bar{1}$	[(HT-$P_2O_6H_2$)Cu(H_2O)(phen)]$(NO_3)_2$·H_2O	1.0	−95.0	$P2_1/n$
(HT-$P_2O_6H_2$)Cl	−2.7	−93.1	$P2_1/c$	(HT-indole-3-propionate)$(ClO_4)_2$·CH_3OH	4.4	−75.9	$P\bar{1}$
(HT-P_2O_6H)·$4H_2O$ (A)	−5.3	−85.8	$P\bar{1}$	catena-((T)-Cd$(SCN)_3$)	0.2	−80.1	$P\bar{1}$
(B)	−20.4	−100.5					
S Form							
(1) C(2)-Substituted (Oxy)thiamin, S							
(phosphalactylHT)Cl·$3H_2O$	−99.2	−173.6	$P2_1/c$	Hg(2-(α-hydroxybenzyl)T)Cl_3·H_2O	−100.4	172.7	$P2_1/n$
[2-(α-hydroxyethyl)HT]Cl_2	−100.3	−145.6	$P\bar{1}$	[2-(α-hydroxybenzyl)oxyHT]Cl_2·$3H_2O$	−92.8	165.9	$P2_1/c$
[2-(α-hydroxybenzyl)HT]Cl_2·$3H_2O$	−92.7	167.2	$C2/c$	(T thiothiazolone)·$^1/_2CH_3COCH_3$	−82.9	176.1	$P2_1/c$
(2) Free Thiamin Salt, S′							
(HT)$(HgCl_4)$·H_2O	−103.0	179.0	$P2_1/c$	(HT)$(CoCl_4)$·H_2O	−111.6	−135.5	$P2_1/n$
(HT)$(CdCl_4)$·H_2O	−110.4	−137.3	$P2_1/n$	(T)(picrate)	−99.3	−122.9	$P\bar{1}$
(3) Free Thiamin Coordinated to N(1′), S′							
Cd(T)Cl_3·$0.6H_2O$	−112.6	−129.8	$C2/c$	Co(T)Cl_3·$0.4H_2O$	−111.8	−128.9	$C2/c$
Zn(T)Cl_3·$0.4H_2O$	−113.4	−130.4	$C2/c$	Zn(T)Br_3·$0.2H_2O$	−113.5	−130.5	$C2/c$
V Form							
(1) Oxythiamin (Substituted at C(4′))							
(oxyHT)Cl_2·H_2O (A)	105.5	−62.9	$P2_1/n$	(oxyT)Cl_2·H_2O	103.4	−64.6	$P\bar{1}$
(B)	101.5	−64.2					
(2) C(2)-Substituted Thiamin							
(T thiazolone)	104.1	−74.2	$P2_1/n$				

[a] T represents unprotonated thiamin and HT represents thiamin protonated on N(1′).

negative and N(3) slightly negative (nearly neutral) charges. We also performed an MNDO[30] calculation which shows positive (0.404) and negative (−0.187) charges for S(1) and N(3), respectively. These results are consistent with an ab initio computation for the 2-(α-hydroxyethyl)-3-methylthiazolium ion in which S(1) has positive (0.459) and N(3) negative (−0.187) charges.[18b] The positive nature of the S atom in the thiazolium moiety has been implicit in numerous crystal structures in which S(1) is nearly always involved in electrostatic interaction(s) with a negative atom or ion(s).[9] It has been noted that resonance structures in which the positive charge resides on S(1) but N(3) is neutral can be drawn for both the thiazolium ring and the ylide (II).[1b,c,29a] Rigorous studies seem to be required to elaborate the electronic structure of thiamin. The bond lengths obtained in AM1 calculations show maximum differences of 0.05 Å [N(1′)–C(2′) in protonated thiamin] and 0.06 Å [C(4′)–N(4′α) in unprotonated thiamin] from the average dimensions observed in the crystal structures.

Conformational Potential Energy Surfaces

Thiamin. The MM conformational potential energy map of thiamin is presented in Figure 3a. There are substantial differences between the present relaxed map and Jordan's rigid map.[18a] The F, S, and V regions are energetically similar, while the conformational space for each region is different. When the crystal conformations of free thiamin are superimposed on the map, there are two clusters roughly centered at the F and S′ regions with the single exception of thiamin·$HgCl_4$·H_2O which is situated in the S region. Interconversions of the F and S forms can occur through a concerted rotation of the two torsion angles in two directions, but there is virtually no energy barrier in one of the directions. The small V region is isolated from the others by an energy barrier higher than 2 kcal/mol. It has been suggested that there is an attractive interaction between the acidic H(2) and the π electrons in the pyrimidine ring.[31] This kind of interaction can be considered to be a hydrogen bond in which the aromatic ring acts as the acceptor, and its energy is estimated to be ca. −1 kcal/mol.[32] Therefore, the energy of the F form may actually be lower than the MM value since in the present

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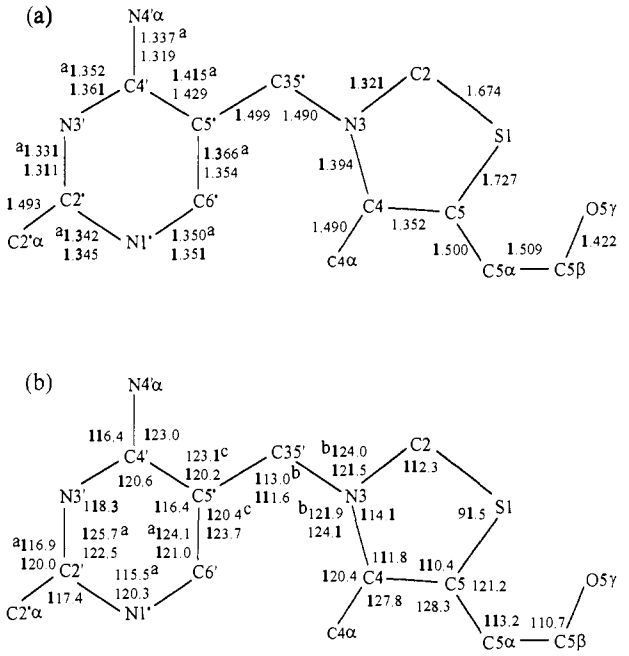

Figure 1. Average (a) bond lengths (Å) and (b) bond angles (deg) in thiamin showing variations depending on the protonation state and the conformation. [a](top) unprotonated N(1′) plus metal-coordinated N(1′), (bottom) protonated N(1′); [b](top) F form, (bottom) S′ form; [c](top) F form plus S′ form, (bottom) S form. A single average value is given when there is no variation.

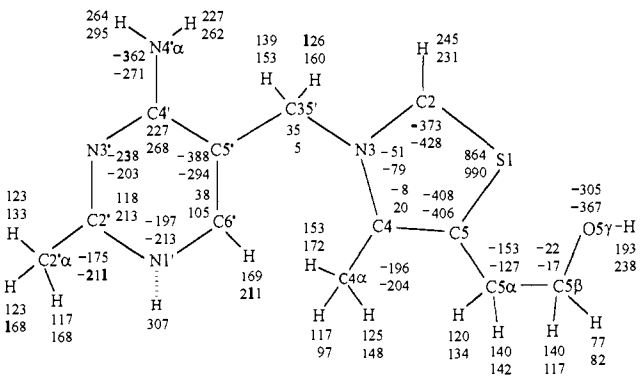

Figure 2. AM1 atomic charges (×10³) in unprotonated (top) and protonated (bottom) thiamins.

calculations this attractive interaction was not treated as such but rather as a normal van der Waals interaction. The presence of the H(2)-π attractive interaction is circuitously supported by the fact that the global minimum ($\phi_T \approx \pm 40°$ and $\phi_P \approx \pm 65°$) is different from the clustered crystal conformations ($\phi_T \approx 0°$ and $\phi_P \approx \pm 90°$) in which its energy can be maximized.

4′-Deaminothiamin, Oxythiamin, and Pyrithiamin. The energy maps of 4′-deaminothiamin, oxythiamin, and pyrithiamin, which are strong antagonists of thiamin, are presented in Figure 3b–d. Since thiamin analogues without a chiral C(2) substituent are centrosymmetric, only half of each map is unique. For 4′-deaminothiamin, only a quarter of the map is unique since the 2′-methylpyrimidinyl moiety is symmetric with respect to the thiazolium moiety. The gross features of the energy maps at 6 kcal/mol level are similar to each other, but the details inside the low-energy regions are quite different. The 4′-deaminothiamin map is more like the oxythiamin than the thiamin map. The crystal structure of 4′-deaminothiamin has not been determined but those of its structural congeners benzylthiamin chloride (**10**)[16a] and 3-benzyl-4-methylthiazolium bromide[16b] have been. The global minimum coincides with the crystal structures (which can be classified as either V or S due to symmetry), and there is virtually no energy barrier between the various conformers. The oxythiamin map is quite different from the thiamin map in the overall shape, and the global minimum V form is exactly the same as the crystal V comformation.[14] The pyrithiamin map is very similar to the thiamin map except that the energy barrier to the V form becomes higher by 1 kcal/mol. As in the case of thiamin, the global minimum, although in the F region, is different from the crystal F conformation of amprolium (**11**), a structural congener of pyrithiamin.[33] The crystal structure of the latter has not been reported yet.

Thiamin Thiazolone and Thiamin Thiothiazolone. Both compounds were originally proposed to be the transition-state analogues[34] of thiamin although strong evidence found later indicated that these are intermediate analogues.[35] The energy maps of TT (Figure 3e) and TTT (Figure 3f) show a clear difference in the conformational properties of these congeneric compounds. The TT map (Figure 3e) shows a distinct global minimum ($\phi_T = \pm 95°$, $\phi_P = \mp 75°$) which agrees well with the crystal V conformation with an N–H···O intramolecular hydrogen bond.[15] In TTT, the global minimum also appears in the V region ($\phi_T = \pm 130°$, $\phi_P = \mp 45°$). However, it is not so distinct as in TT, and only 1 kcal/mol lower in energy than the other minima, even though we have used the most favorable parameters for the formation of the N–H···S hydrogen bond in order not to underestimate its energetic contribution.[23] The crystal S conformation occurs in the center of the widest S region. Interconversion of the V and S forms should be facile due to the low-energy barrier. In both maps, the F form is located at a saddle point indicating that it becomes an energetically unfavorable form for thiamin analogues with a C(2)-substituent larger than a H atom.

6′-Methyl-4*H*-thiamin and 6′-Methylthiamin. 6′-Methyl-4*H*-thiamin shows partial activity (ca. 20%) but 6′-methylthiamin shows no activity in PDC. These data were the crucial ones that led Schellenberger to propose the V as the active form of thiamin.[3a] The crystal structures of both compounds have not been determined. The 6′-methyl-4*H*-thiamin map (Figure 3g) shows two distinct F and V + S′ minima whose centers locate at ($\phi_T = 0°$; $\phi_P = \mp 90°$) and ($\phi_T = \pm 180°$; $\phi_P = \mp 90°$), respectively. This symmetric nature occurs due to the steric equivalence of C(2) and C(4) both of which are bonded to the H atoms. Both regions are nearly isoenergetic and occupy similar space, although the energy of the F form might be slightly lower as discussed in thiamin. The 6′-methylthiamin map (Figure 3h) shows a wide F and two narrow V ($\phi_T \approx \pm 135°$; $\phi_P \approx \mp 50°$) and S′ ($\phi_T \approx \pm 130°$; $\phi_P \approx \pm 130°$) minima with the energy barriers of ~5 kcal/mol. The V form is 1 kcal/mol higher in energy than the F and S′ forms. In both compounds, S forms with $\phi_P \approx 180°$ are no longer stable.

2-(α-Hydroxyethyl)thiamin and Lactylthiamin. There are four major rotatable bonds [N(3)–C(35′), C(35′)–C(5′), C(2)–C(2α), C(2α)–C(2β)] in LT and three in HET. It is generally difficult to obtain smoothly contoured 2-D energy maps for compounds with many rotatable pendant groups such as disaccharides due to the multiple minimum problem.[36] The 2-D energy map usually shows discontinuous contours with a sudden drop in the energy when it is calculated with respect to a specific conformer of the pendant group(s). One way to circumvent this problem is to make a composite map from several maps each of which is calculated with the different local minima conformer(s) of the pendant group(s) as the starting conformer. Our unique iterative four-way scanning method developed for such a system makes it

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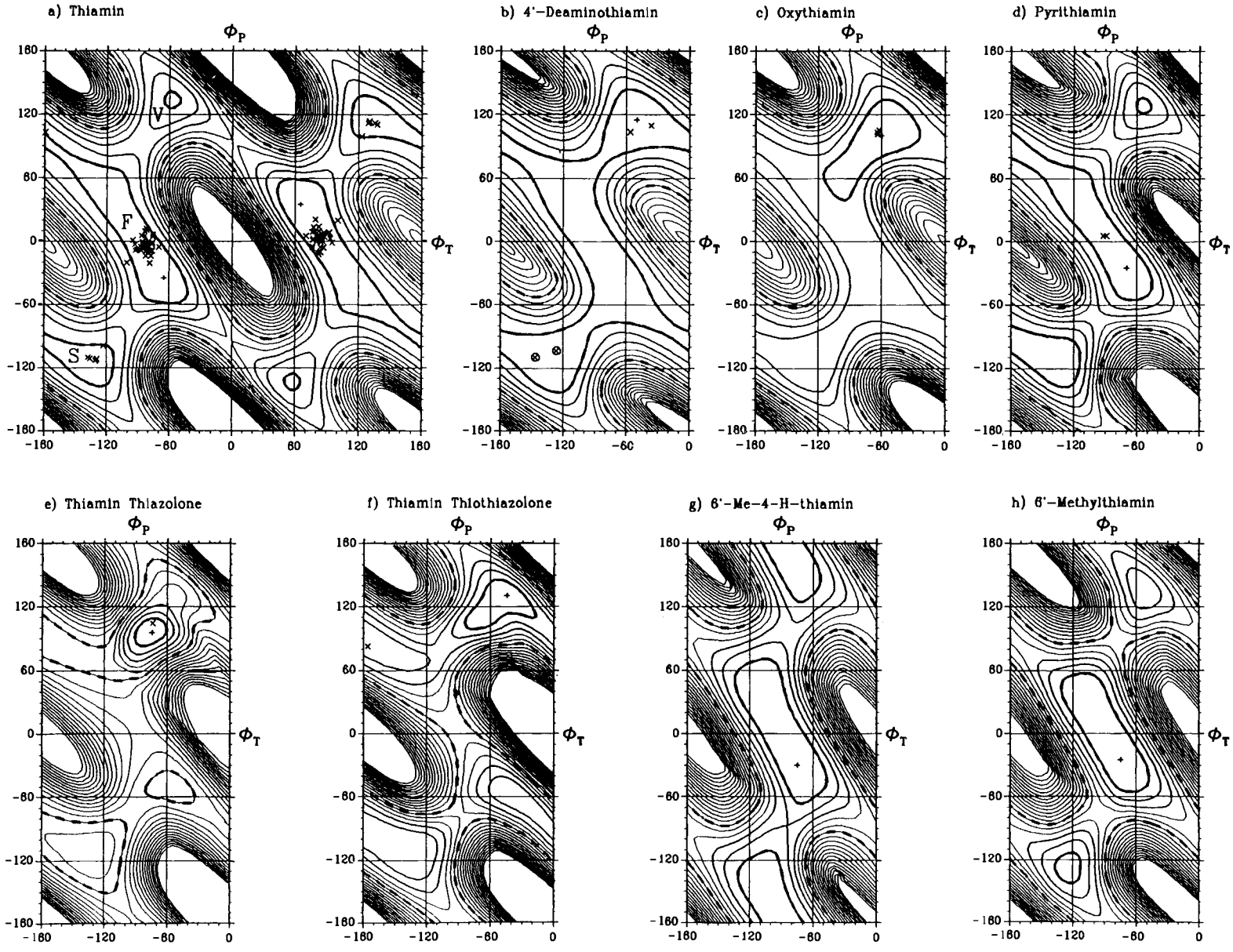

Figure 3. The relaxed MM conformational potential energy maps of thiamin and its analogues. The contour level is 1 kcal/mol and the lowest 20 kcal/mol levels are drawn for each map. The dashed thick lines denote the 6 kcal/mol level from the global minimum. Inside the low-energy region, the contour lines become thicker the lower the energy. The + and × signs denote the global minimum and the crystal conformation, respectively. The approximate locations of the F, S, and V forms are depicted in (a). The circled × signs in (b) represent the same molecules as those without the circle, reflecting the symmetric nature of the molecule.

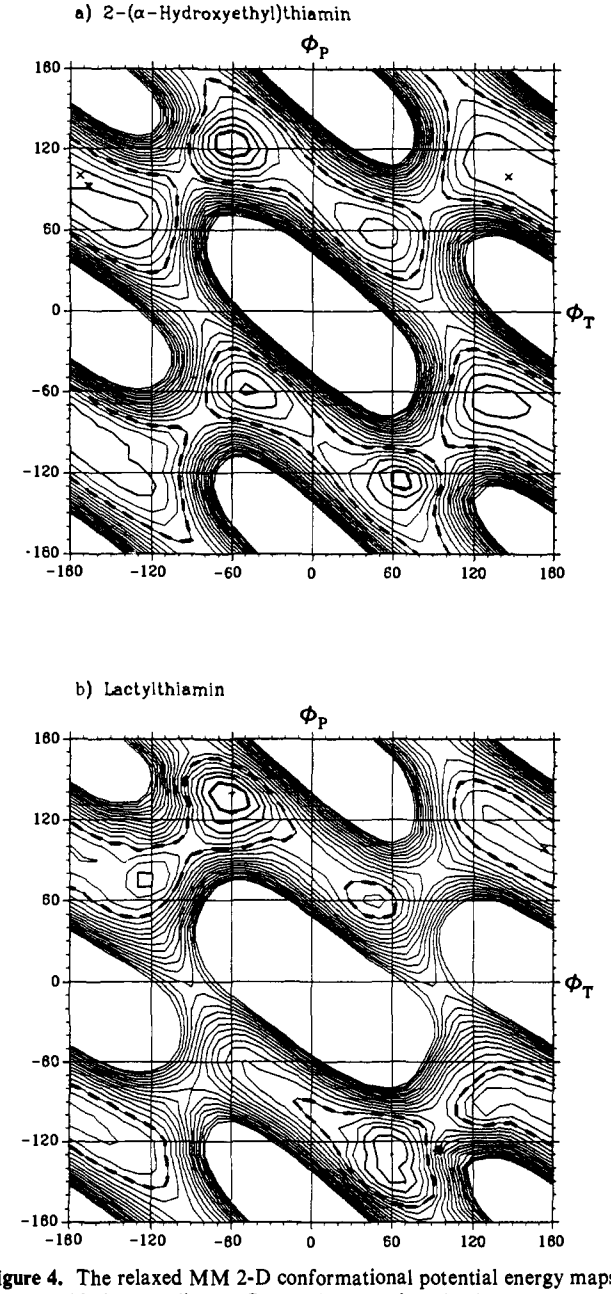

Figure 4. The relaxed MM 2-D conformational potential energy maps of the stable intermediates. Contouring was done in the same way as stated in Figure 3. The crystal structures of HET and its analogues including 2-(α-hydroxybenzyl)oxythiamin are marked in (a) and the crystal structure of PLT is marked in (b).

possible to obtain a smooth contour map starting from one arbitrary side-chain conformation in an automated single run of calculation. In the resulting map, the side-chain conformations may be different for each grid point. This efficient method gave better results for carbohydrates than the other commonly used methods.[19,36]

The relaxed 2-D energy maps of HET and LT in terms of ϕ_T and ϕ_P are presented in Figure 4a,b, respectively. The absolute configuration of the chiral C(2)-substituent of HET was fixed as R according to results of Kluger et al.[37] The absolute configuration of LT was arbitrarily fixed as S, which assumes that the stereochemistry is retained rather than inverted when the enamine (V) is converted into HET. The present energy maps of LT and HET are not centrosymmetric, and the crystal structures correspond to only one location in the present maps even though there are enantiomers in the crystal structures with centrosymmetric space groups. As pointed out by Turano et al., the introduction of a chiral substituent on C(2) generates a second element of asymmetry by virtue of restricted rotation about the bonds at C(35′).[13] They also noted that the conformation of thiamin in the synthetic reaction is a stereochemical determinant of the resultant product producing essentially two of four possible isomers, since a specific enantiomeric form at C(2α) exists only with a specific "enantiomeric" form at C(35′).

Jordan's HET map showed six distinct local minima separated by more than 18 kcal/mol energy barriers.[18b] There are also six minima in the present relaxed map, but they are separated with much lower energy barriers (at most $< \sim 9$ kcal/mol). The global minimum is in the V(+ −) region, where the signs in the parentheses denote those of the torsion angles ϕ_T and ϕ_P. There are two wide S regions representing the two enantiomeric S forms. The crystal structures of HET[9] and its analogues[10-12] are in the S(+ +) and S(+ −) regions. The relaxed conformational energy map of LT is the first one ever obtained for this intermediate. The gross features of the LT map are similar to those of HET with the global minimum in the V(+ −) region. However, a local minimum at $\phi_T \approx -60°$, $\phi_P \approx -50°$ in HET disappeared in the LT map, and a narrow S(+ −) local minimum became isolated. The available conformational space in LT is narrower and the energy barriers between the conformers is higher than in HET since the size of the LT substituent is bigger. PLT is the only analogue of LT whose crystal structure has been determined.[13] It is located at the center of the S(+ −) region.

6′-Methyl-4*H*-lactylthiamin and 6′-Methyllactylthiamin. Energy maps of these compounds were obtained to test the validity of Schellenberger's interpretation of the catalytic properties of 6′-methyl-4*H*-thiamin and 6′-methylthiamin. The crystal structures of these compounds have not been determined. There are large differences in the S regions between the maps of these compounds and LT. The 6′-methyl-4*H*-lactylthiamin map (Figure 5a) shows that the two V regions are wider than those in the LT map with more rotational freedom in ϕ_T reflecting the conformational property of its parent compound. The global minimum V(+ −) form is lower in energy by 1 and 4 kcal/mol than the V(− +) and S(+ −) forms, respectively.

The 6′-methyllactylthiamin map (Figure 5b) shows several isolated minima separated by high-energy barriers (<13 kcal/mol), indicating that the molecule is sterically very congested. Actually this compound is the sterically most congested one among the thiamin compounds since all of the C(2), C(4), C(4′), and C(6′) positions, that are critical sites determining the conformational characteristics of the molecule, have substituents bigger than a H atom. High-energy barriers indicate that the conformational transition between the conformers would be prohibitive under normal conditions. The V(+ −) form is again the global minimum and it is 2 kcal/mol lower in energy than the V(− +) form. The global minimum conformer shown in Figure 6 has an N–H···O(2α) hydrogen bond and a close contact between H(35′) and O(2α) without any unfavorable interactions. The latter interaction can be regarded as a C–H···O type of hydrogen bond as observed in the crystal structure of PLT.

Local Minima Structures of HET and LT

The individual local minima conformers, neglecting the differences in the orientation of the hydroxyl and methyl groups, have been identified using the systematic grid search method. There are 23 and 26 unique minima for HET and LT, respectively, within 6 kcal/mol above the global minima. There are only three representative low-energy conformers for the 2α-hydroxyethyl and 2α-lactyl side chains as shown in Figure 7a,b, respectively.

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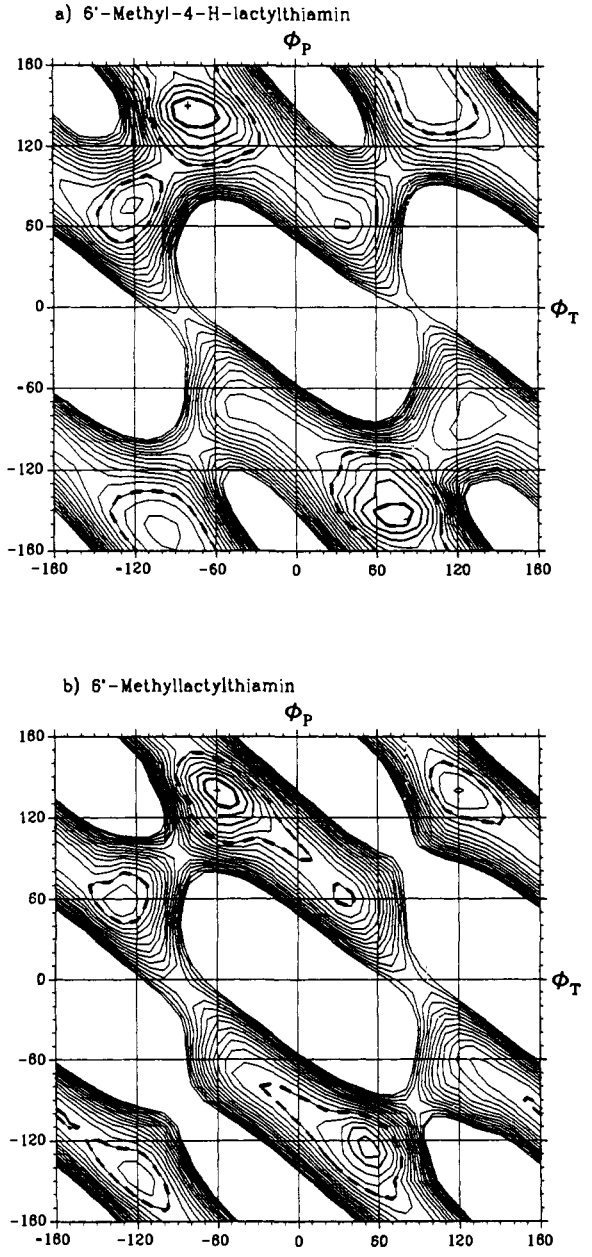

Figure 5. The relaxed MM 2-D conformational potential energy maps of the lactylthiamin analogues. Contouring was done in the same way as stated in Figure 3.

It has been found that the crystal conformations of the substituents of HET[9] and PLT[13] are different although the thiamin portions have similar S conformations. In HET O(2α) is close to the thiazolium S atom. In contrast O(2α) is hydrogen-bonded to the bridging methylene H atom and the bond to the phosphonate is oriented perpendicular to the thiazolium ring plane in PLT. Therefore, the crystal conformations of the substituent in HET and its analogues correspond to conformation A of HET while that of PLT corresponds to conformation B of LT in Figure 7. The torsion angles, the types of C(2) side-chain conformation and the hydrogen bond for the local minima conformers are listed in Table II. The V(+ −)B and V(+ −)A forms are the global minima for HET and LT, respectively. The two lowest energy V and one higher energy S conformers of HET have the N–H···O-(2α) hydrogen bond. The crystal conformation of HET, which is not detected as a local minima, corresponds to the S(+ +)A conformer which is higher in energy by 3.1 kcal/mol than the global minimum V(+ −)B conformer. The seven lowest energy V and S conformers of LT have intramolecular hydrogen bonds between the 4'-amino N atom and either the carboxyl or the

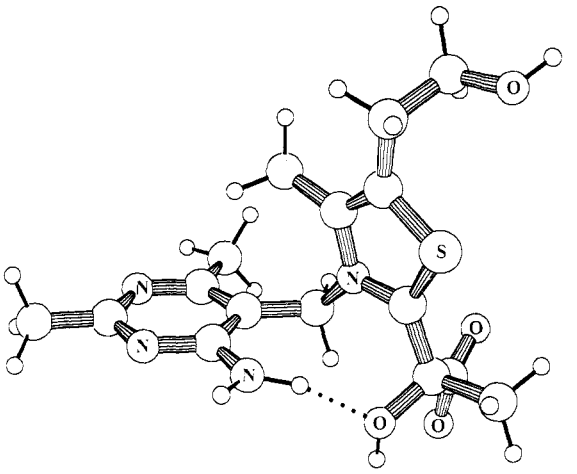

Figure 6. Global minimum structure of 6'-methyllactylthiamin in the V form. The dotted line denotes the hydrogen bond.

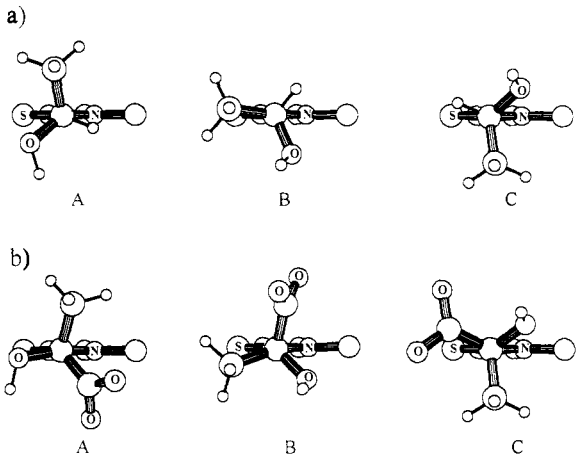

Figure 7. Representative local minima structures of the C(2) substituents in (a) HET and (b) LT. All plots were drawn with the same viewing direction along the C(2)–C(2α) bond.

hydroxyl O atom. The crystal conformation of PLT corresponds to the S(+ +)B conformer which is higher in energy by 5.1 kcal/mol than the global minimum V(+ −)A conformer. Apparently similar conformers, especially in the S region, are detected as independent local minima, indicating that the potential energy surfaces of both compounds are more complex than appeared in the relaxed energy maps. The superimposed stereo plots of the conformers with the N–H···O hydrogen bond of HET and LT together with the crystal structures of HET and PLT are shown in Figure 8a,b, respectively.

Discussion

The present study makes it possible to answer several important questions about the structural characteristics and conformational properties of thiamin-related compounds and to suggest a plausible stereochemical model of thiamin catalysis.

Structural Characteristics and Conformational Properties of Thiamin. The variations in the exocyclic bond angles around N(3) and C(5') can be explained from a consideration of the conformational effects on the bond angles. The two angles at N(3) would primarily be determined from the balance of the two 1,4-interactions between C(35') and two substituents at C(2) and C(4) which are conformation-independent since the involved atoms are in a plane. If the size of the substituent at one ring atom is larger, the exocyclic angle involving the ring atom would be widened to minimize the possible steric repulsion between C(35') and the substituent. Therefore, we can expect that C(4)–N(3)–C(35') should be larger than C(2)–N(3)–C(35') for free

Table II. Types of Conformation and Hydrogen Bond Acceptor, Torsion Angles (deg), and Relative Energy (kcal/mol) for Local Minima of HET and LT

no.	conf type	H-bond	ϕ_T	ϕ_P	$\phi_{2\alpha}$[a]	ΔE
			(a) HET			
1	V(+ −)B	OH	125.8	−62.9	−71.6	0.00
9	V(+ −)A		129.7	−56.9	−159.8	2.69
14	V(+ −)C		138.6	−54.2	41.4	3.55
2	V(− +)C	OH	−124.9	64.3	50.0	0.66
6	V(− +)A		−140.3	55.3	−148.7	2.60
19	V(− +)B		−128.0	56.2	−80.1	3.98
21	V(− +)B		−131.3	56.5	−63.5	4.08
3	S(− +)A		−66.5	131.8	−135.3	1.42
12	S(− +)C		−90.9	170.5	38.3	3.37
13	S(− +)B	OH	−72.0	114.2	−54.2	3.52
4	S(+ −)A		73.2	−143.6	−141.6	1.70
17	S(+ −)C		70.7	−132.6	33.4	3.65
24	S(+ −)B		90.9	−175.5	−61.1	4.19
5	F(− −)A		−56.8	−53.0	−133.7	1.78
22	F(− −)B		−56.5	−51.1	−62.5	4.11
26	F(− −)C		−73.1	−33.1	40.5	5.87
7	S(− −)A		−125.4	−134.6	−155.0	2.62
10	S(− −)A		−108.5	−159.9	−160.8	2.79
8	F(+ +)A		58.1	47.5	−118.2	2.63
11	F(+ +)A		59.9	47.4	−148.5	3.16
20	F(+ +)B		54.8	50.6	−96.1	4.00
25	F(+ +)C		60.0	51.0	30.4	4.42
15	S(+ +)C		123.0	136.9	47.7	3.59
16	S(+ +)C		108.3	158.1	53.3	3.62
18	S(+ +)B		122.8	131.1	−82.1	3.78
23	S(+ +)B		105.9	155.4	−62.1	4.18
			(b) LT			
1	V(+ −)A	COO	141.8	−61.8	−177.0	0.00
4	V(+ −)B	OH	124.5	−64.9	−58.6	1.94
13	V(+ −)C		139.1	−53.7	37.3	4.20
2	V(− +)B	COO	−146.3	65.1	−41.0	0.88
3	V(− +)C	OH	−124.1	63.0	45.5	1.30
8	V(− +)A		−138.4	53.5	−164.4	3.91
5	S(− +)A	COO	−83.5	133.6	−168.0	2.94
9	S(− +)A		−97.4	178.6	−177.9	3.91
11	S(− +)C		−89.8	167.9	34.2	4.08
6	S(+ −)B	COO	78.5	−127.4	−57.9	3.06
7	S(+ −)C	OH	77.9	−117.0	33.0	3.74
14	S(+ −)A		97.1	−174.1	−165.4	4.28
19	S(+ −)B		87.8	−167.2	−32.3	5.01
10	S(− −)A		−124.1	−136.8	−169.8	4.03
12	S(− −)A		−110.1	−158.1	−175.8	4.18
16	S(− −)A		−101.3	−173.2	−177.8	4.37
17	S(− −)C		−114.5	−140.2	42.4	4.38
23	S(− −)B		−122.4	−130.5	−2.2	5.94
15	S(+ +)A		126.1	135.3	−175.0	4.28
18	S(+ +)C		104.8	165.9	49.9	4.41
20	S(+ +)B		91.8	179.4	−33.7	5.05
22	S(+ +)A		104.1	173.2	−166.2	5.90
21	F(+ +)C		61.5	49.1	25.3	5.06

[a] N(3)–C(2)–C(2α)–O(2α).

thiamin since the 4-methyl group is larger than H(2). By the same rationale, C(4′)–C(5′)–C(35′) should be larger than C(6′)–C(5′)–C(35′) for both free and C(2)-substituted thiamins since the 4′-amino group is larger than H(6′). The average values of C(4)–N(3)–C(35′) and C(4′)–C(5′)–C(35′) are indeed larger by 2.6° and 2.7° except for the following two cases: C(2)–N(3)–C(35′) is larger by 2.1° than C(4)–N(3)–C(35′) only for free thiamin in the F form, as noted by Cramer et al.,[31] and C(6′)–C(5′)–C(35′) is larger by 3.5° than C(4′)–C(5′)–C(35′) only for C(2)-substituted thiamins and thiamin·$HgCl_4$·H_2O in the S form, as observed in this study. These exceptions are the results of secondary 1,4-interactions that become conditionally operative depending on the conformation. In the F form, C(2), N(3), C(35′), and C(5′) become approximately coplanar and thus the 1,4-interaction between C(2) and C(5′) becomes more dominant than that between C(35′) and H(2), making the C(2)–N(3)–C(35′) angle larger. In the S form, N(3), C(35′), C(5′), and C(6′) become coplanar, and thus the 1,4-interaction between N(3) and C(6′) makes the C(6′)–C(5′)–C(35′) angle larger. The largest difference of 10° has been observed in 2-(α-hydroxybenzyl)-oxythiamin.[12] In contrast, for free thiamins in the S′ form, C(4′)–C(5′)–C(35′) is larger than C(6′)–C(5′)–C(35′) since C(6′) is directed toward C(2) rather than N(3) in this form with $\phi_P \approx$ 130° and thus the conformation-dependent 1,4-interaction does not affect the bond angles. The variations in the exocyclic bond angles in thiamin are merely an example of the general notion that the bond angles, but usually not the bond lengths, are subject to the influence of the molecular conformation. Differences in one ring system do not affect the property of the other ring as far as the molecular dimensions are concerned, indicating the absence of long-range inductive effects between the two aromatic ring systems.

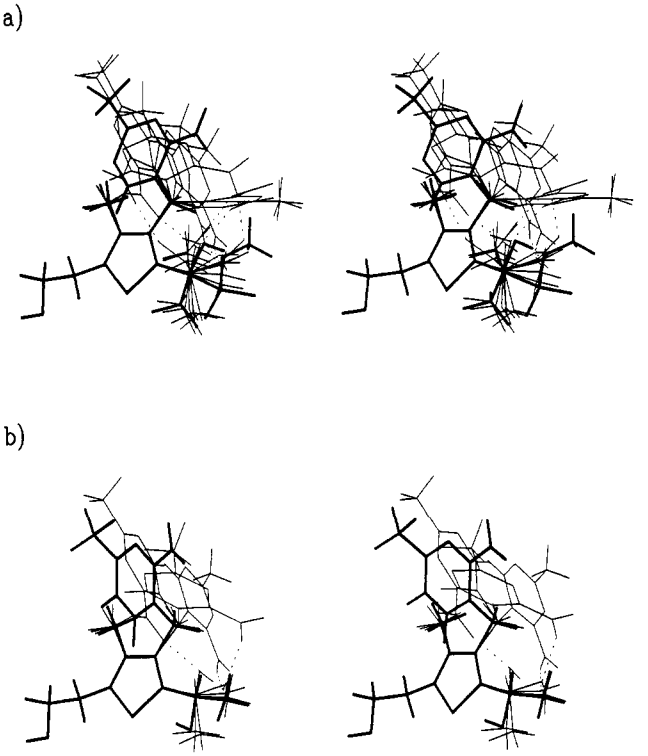

Figure 8. The stereoscopic drawing of the superimposed conformers with the N–H···O hydrogen bond for (a) LT and (b) HET. The dotted line denotes the hydrogen bond. The molecules in thick line are the crystal structures of (a) PLT and (b) HET.

Classification of crystal conformations shows that, except for the cases of free thiamin in the S or S′ form, the chemical perturbations at C(2) or C(4′) of free thiamin cause a conformational change from F to S or V but those at other positions such as N(1′) or O(5γ) do not. Therefore, the crystallographic data are in good agreement with our early notion that the conformations of thiamin and its analogues are determined by intricate balances of the short-range intramolecular interactions, especially between the substituents at C(2), C(4), C(4′), and C(6′) which are involved in the 1,4-interactions with the methylene bridge C(3,5′).[15] The structural difference between TT and TTT adds substantial support for this notion since it involves only one congeneric atom at C(2). Conformational analyses also provide a quantitative explanation for this notion.

The relaxed conformational energy map of thiamin shows that all of the F, V, and S forms of thiamin are intrinsically stable in vacuum. However, their populations in solution would be different. The Boltzmann existence probabilities based on the present MM energy values are 49.7, 36.7, and 13.6% for the F, S, and V forms, respectively. If the presence of the attractive H(2)–π interaction is taken into account, the probability of F would become higher. As it occupies the widest conformational

space and possesses the highest existence probability, the F form can be regarded as the global minimum. Accordingly, the crystal F form is not an artifact of the crystal packing forces. The existence probability of S is still substantial, and the frequency of observing S forms in the crystalline states may be accounted for on statistical grounds. It is well-known that both C(2) and the 4′-amino group are involved in hydrogen-bonding interactions with electronegative atoms or ions. The S or S′ form of free thiamin seems to be induced when the proper interaction modes between the polyhalogenicmetal anions and these hydrogen-bonding sites of the thiamin cation can be established in the crystal lattice. Cramer et al. proposed that there is a correlation between the size of the anion and the conformation based on the fact that the nonbonding distance between N(4′α) and C(2) is longer in S than in F; that is, the F form is favored by smaller metal anions and the S form by the larger ones.[38] This proposal however does not always hold and Aoki et al. recently proposed that the "one-point" and "two-point" halide bridges are responsible for the F and S forms, respectively.[39]

The probability for thiamin to assume the V form in solution should be much lower than the intrinsically low existence probability suggests, because only one 4′-amino H atom is available for intermolecular hydrogen bonds in the V form, whereas two H atoms are available in the F and S forms. Furthermore, conformational interconversion involving the dead-end V form may not be so facile as that between the F and S forms due to the presence of the energy barrier. Therefore, we may conclude that there is practically no chance that free thiamin will crystallize in the V form from solution. However, thiamin can assume the V form when the appropriate environment is provided through various interactions as observed in protein crystal structures. Specifically, it has been observed in PDC that the V form is stabilized by strong van der Waals interactions with the side chain of an isoleucine residue which is wedged tightly between the two aromatic rings.[7] The present study suggests that one of the major functions of the TDP-dependent enzymes is to fix thiamin in the active V form which may not be obtained in solution. Once thiamin becomes the ylide losing a proton on C(2), then the V form becomes more stable due to the $^{\delta+}$H(4′α)···C(2)$^{\delta-}$ electrostatic interaction as confirmed by Friedemann et al.[18c]

Conformational and Binding Properties of Thiamin Antagonists. Conformational analyses of various antagonists provide an understanding of their binding properties for the TDP-dependent enzymes. The V forms of 4′-deaminothiamin, oxythiamin, TT, and TTT either are intrinsically the most stable or have higher existence probabilities than thiamin. One exception is pyrithiamin whose conformational energy map is very similar to that of thiamin. For 4′-deaminothiamin and oxythiamin, all conformers are freely accessible through concerted rotations of ϕ_T and ϕ_P. However, the 4′-amino group imposes energy barriers between various conformers in other compounds suggesting its role as a conformational determinant. The energy maps are in general of good quality as judged from the fact that the crystal structures are consistent with the global minima.

The conformational difference of congeneric TT and TTT exemplifies the intricacy involved in the conformational properties of thiamin-related compounds. TT and TTT were originally proposed to be transition-state analogues of thiamin due to their structural resemblance to the enamine intermediate and their high binding affinities for pyruvate dehydrogenase.[34] However, Kluger et al. have suggested that these are the intermediate analogues and their high affinities are mainly due to their overall neutral charges.[35a] We have determined the crystal structure of TT which shows the V form with an intramolecular N–H···O hydrogen bond. We suggested that this conformational characteristic of TT contributes to the high binding affinity, and we suggested accordingly that the enzyme-bound conformer of thiamin may be the V form.[15] We also determined the crystal structure of TTT which revealed the S form.[17] This was an unexpected result because it is quite tempting to speculate that TTT may easily assume a similar V form with a comparable intramolecular N–H···S hydrogen bond. Detailed analysis of the MM results shows that the N–H···S hydrogen bond in TTT does not provide as significant an energetic contribution as N–H···O does in TT because its strength is intrinsically weaker. Furthermore, the possible steric repulsion between H(C6′) and C(4α), which originates from the longer C=S bond and the larger radius of the S atom, prohibits the formation of a hydrogen bond with a decent geometry. In TTT, the existence probabilities for the V and S forms and the small local minimum near F are 54.2, 41.7, and 4.1%, respectively. Although the energy of the global minimum V form is 1 kcal/mol lower than that of the S form in the present calculation, the actual energy of the V form might be slightly higher than the present value since we might have overestimated the energy of the N–H···S hydrogen bond.[23] Whether the V form is the global minimum for TTT or not, its existence probability is still higher than that of the V form of thiamin. Thus our proposal concerning conformational effects to the high binding affinity of TT still holds for TTT.

Both the catalytic activity and the binding property of the thiamin analogues vary depending on the enzymes. 6′-Methylthiamin is inactive and 6′-methyl-4*H*-thiamin is partially active in PDC as stated above. However, the former compound is active and the latter inactive in transketolase.[40] 4′-*N*-Methylthiamin diphosphate also shows a difference in activity, that is, inactive in PDC but active in transketolase.[40] The diphosphate ester of TT binds 20 000 times more strongly than TDP does for *E. coli* pyruvate dehydrogenase,[34] and only 30 times more strongly for *E. coli* pyruvate oxidase[41] but does not bind particularly tightly to yeast transketolase.[42] Schellenberger et al. found that *N*(1′)-pyridyl-TDP is active but *N*(3′)-pyridyl-TDP and 4′-deamino-TDP are inactive in PDC, pyruvate dehydrogenase, and transketolase, while *N*(1′)-pyridyl-TDP and 4′-deamino-TDP bind to all three apoproteins but N(3′)-pyridyl-TDP binds only to transketolase.[3d] There are common features in the binding mode of thiamin in the three protein crystal structures although a detailed comparison has not been reported yet. For example, in each of the three proteins, a hydrophobic group is located such that it keeps the cofactor in the V form, and N(1′) is hydrogen bonded to a glutamic O atom, and the pyrophosphate moiety of the cofactor is bound in the same manner to the protein utilizing the same side chains and a Mg ion. On the other hand, there are also differences in the binding mode of thiamin. Notably, the 4′-amino group is involved in two hydrogen bonds (a histidine N and a main chain O atoms) in transketolase and in one (a main chain O atom) in PDC, but it is not hydrogen-bonded in pyruvate oxidase. The data above also indicate that there are subtle differences in the active sites of the proteins.

Schellenberger's active V model was originally based on the assumption of the so-called "steric equivalence" of the 4 and 6′ positions, which he derived from molecular models. He conjectured that the hydroxyethyl adduct of 6′-methylthiamin cannot assume the V form due to steric hindrance between the two methyl groups at the 4 and 6′ positions and thus is inactive, while the hydroxyethyl adduct of 6′-methyl-4*H*-thiamin with only one methyl group can assume the V form and thus is partially active.[3a]

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However, the present study indicates that the 4 and 6′ positions are not sterically equivalent, and thus Schellenberger's assumption may not be correct. The V form of 6′-methyllactylthiamin can exist as the global minimum even though the molecule is sterically very congested. It is interesting to note that the existence probability for V of its parent compound is even lower than that of thiamin. For 6′-methylthiamin, the probabilities are 74.0, 18.4, and 7.6% for the F, S, and V minima, respectively. The fact that 6′-methylthiamin is catalytically active in transketolase suports our result. It is very likely that 6′-methylthiamin is active in transketolase because the presence of the extra 6′-methyl group in the V form does not give rise to any severe adverse steric incompatibilites with the protein. On the other hand, 6′-methylthiamin is inactive in PDC because it may not be able to assume the V form owing to steric hindrance between the 6′-methyl group and the protein. We may have to consider not only the conformational properties of the cofactor or the ligand molecule itself but also its interaction mode with the protein for interpretation of the biochemical data. The interaction mode and binding process of each analogue may be different for each protein. Kluger already has found that the binding of the diphosphate ester of TT in PDC is a complicated process.[35a]

Active Conformations of the Intermediates. The conformational energy maps show that the V forms are the global minima for both LT and HET. The detailed geometric features were obtained from the local minima structures identified in the systematic grid search. Thus far, five crystal structures of C(2)-substituted thiamin-related compounds have been reported (see Table I). All of them crystallized in the S form instead of the global minimum V form. This phenomenon can be rationalized as follows: Free thiamin in solution would exist almost exclusively in either the F or S form with almost none in the V form. Accordingly, the adduct formation in solution would proceed with thiamin in the F or S form. But the F forms of the C(2) adducts are unstable, while the S forms are stable. Therefore, it seems that the thiamin molecules are induced to assume the S form for the reaction to proceed and the product crystallizes in the S form. The V form of the C(2) adducts is more stable than the S form in vacuum mainly due to the intramolecular N–H···O hydrogen bond. However, in the S form, the same amino H atom can be engaged in an intermolecular hydrogen bond in solution and crystal, and thus the total system is not energetically unfavorable. Once adduct formation is completed in the S form, the conformational change to the V form in solution becomes unfavorable because there is an intrinsic energy barrier to be overcome, and the already established intermolecular hydrogen bonding scheme would have to be abolished.

Although the crystal structure of a protein containing an active intermediate has not been reported yet, the observation of the V form of free thiamin inside the protein strongly suggests that the active conformations of the intermediates are also V forms as far as the conformation of the thiamin portion is concerned. It is very unlikely that thiamin would change its conformation from the V form in which an intramolecular N–H···O hydrogen bond can be utilized to lower the energy of the adduct formed. It has been found that the exogenous diphopshate esters of both (+)-HET and (–)-HET can bind to PDC to produce acetaldehyde.[37b] Since the enzyme should bind a specific enantiomorphic form and since HET in solution probably exists in the S form, a conformational change into the active V form seems to have been induced by the protein in spite of the fact that it is very unlikely to take place in solution.

Even if we accept the active V form for the thiamin portion, it is not yet certain which is the active conformer of the intermediates as far as the substituent portion is concerned. Kluger and Pletcher jointly made a proposal that LT would assume the same SB conformation inside the enzyme as observed in the crystal structure of PLT.[13] They elegantly justified the active B conformation for the lactyl substituent considering the electrostatic and steric aspects and showed that the principles of least motion[43] and maximum orbital overlap[44] can be applied to thiamin catalysis. As reasoned above, there may virtually be no probability that the S conformer is the active one for the thiamin portion. However, the active B model is very reasonable as far as the substituent portion is concerned. Since it has been found that thiamin assumes the V form in the enzyme and the global minima conformers of LT and HET are the V form, it is now possible to revise this proposal assuming that *the active conformers of LT and HET are the V(+ –)B forms both of which have the N–H···O(hydroxyl) hydrogen bond.* Only this combination of the V conformers allows us to simultaneously satisfy the two postulates that the 4′-amino group somehow participates directly in the intramolecular acid–base catalysis and that the decarboxylation of pyruvate is subject to the principles of least motion and maximum orbital overlap. These active conformers are the fourth lowest energy and the global minimum conformers of LT and HET, respectively. The energy of the *active* V(+ –)B form of LT is 1.9 kcal/mol higher than that of the global minimum V(– +)A conformer with the N–H···O(carboxyl) hydrogen bond but 3.1 kcal/mol lower than that of the SB model proposed by Kluger and Pletcher. The *active* V(+ –)B form of HET is 3.1 kcal/mol lower in energy than the crystal SA conformer.

A Stereochemical Model for Thiamin Catalysis. A putative stereochemical model for the catalytic mechanism in PDC, based on the assumption of the active V(+ –)B form, is shown in Figure 9. From extensive kinetic data for nonenzymic[45] and enzymic[46] reactions, Schellenberger and Schowen found that the overall catalytic reaction is $10^{12.5}$ times faster in PDC than in aqueous solution with individual acceleration factors of $10^{12.6}$ for the formation of the TDP–pyruvate adduct and $10^{7.1}$ for the decarboxylation.[46a] They also found that the rate constants for the decarboxylation and product release steps are similar but could not obtained an acceleration factor for the product release step due to a lack of nonenzymic data. According to their free-energy diagrams, PDC stabilizes the reactant state preceding TDP addition to pyruvate by 18.2 kcal/mol and the transition state for the addition by 19.8 kcal/mol.[46b] PDC also stabilizes the reactant state preceding decarboxylation (presumably α-lactyl–TDP) by 6.5 kcal/mol and the transition state for decarboxylation by 16.3 kcal/mol. They also suggested a reactant-like structure for the addition transition state from the observation that PDC stabilizes both reactant and transition states by similar amount of energy. We suggest that the intramolecular N–H···O hydrogen bond in the proposed active V conformer is a stereoelectronic factor that contributes to the stabilization of both reactant and transition states for the addition step. This hydrogen bond can also stabilize the polar transition state developed in the final product release step. The formation of this hydrogen bond in the V form should be a unique feature in the enzymic reaction since, as discussed above, it is very unlikely that this hydrogen-bonding interaction can be utilized in the catalytic reaction in solution.

It was once suggested, from the crystal structures of HET and its analogues, that the thiazolium moiety is selected for the coenzyme instead of the other azolium systems such as imidazolium or oxazolium because of the catalytic role of the S atom. S(1) was portrayed to stabilize the transition state through an $^{\delta+}S\cdots O(2\alpha)^{\delta-}$ electrostatic interaction. It was also suggested that

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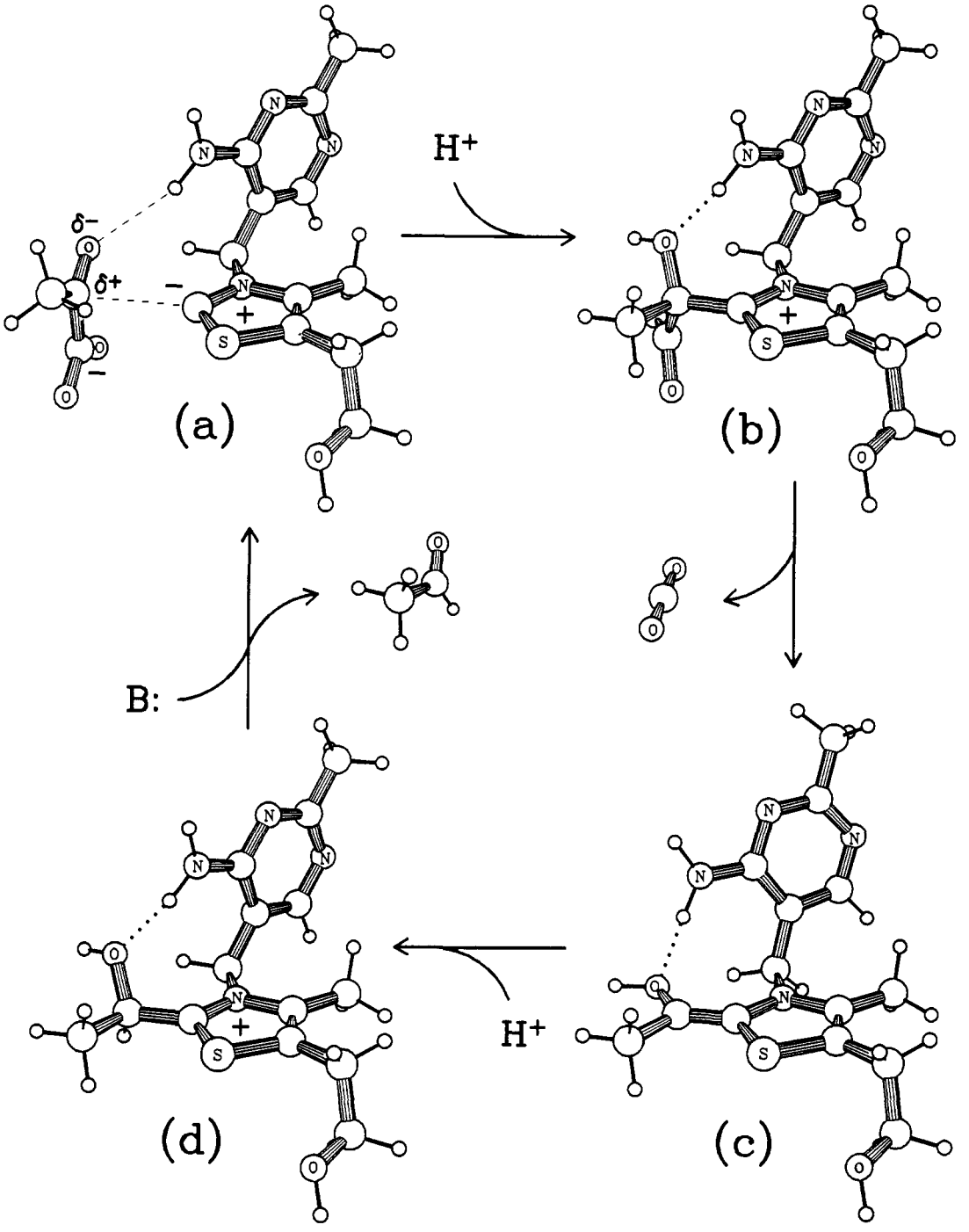

Figure 9. A putative stereochemical model for thiamin catalysis. The molecule in (c) represents either the enamine or α-carbanion intermediate.

the $^{\delta-}$C(2)–S(1)$^{\delta+}$ bond polarity may be important in aligning the $^{\delta+}$C=O$^{\delta-}$ dipole of pyruvate.[18b,47] However, an S···O(2α) interaction is not present in the crystal structure of PLT, and the catalytic role of S(1) was dismissed in the model of Kluger and Pletcher. Instead they suggested that the $^{\delta+}$N(3)···O(2α)$^{\delta-}$ electrostatic interaction would assist in the initial alignment of pyruvate to the thiazolium ylide. We now suggest that the N(4′α)H···O(2α) hydrogen-bonding interaction is a major factor assisting in the alignment of pyruvate to the thiazolium ylide by lowering the energy of the transition state leading to the lactyl derivative. The $^{\delta+}$N(3)···O(2α)$^{\delta-}$ interaction might not be significant since it is subject to the screening effect of the bridging methylene moiety. There is also a possibility that N(3) may not be as positive as formally depicted. Recent AM1 and ab initio calculations[48] for the decarboxylation of lactylthiazolium indicate that the thiazolium ring simply serves as a better electron sink than the others for the decarboxylation process, as Breslow originally proposed.

Our model differs from Schellenberger's[3d] in that an extra acid and/or base other than the 4′-amino group are required, at least, in one stage of the catalytic cycle. During a cycle of thiamin catalysis, proton addition or abstraction is involved in each step. We initially need a base for the formation of the thiazolium ylide at the beginning of the catalysis and then two acids and one base during a catalytic cycle since the thiazolium ylide is recycled. First, an acid is required for the formation of LT from the ylide and the pyruvate anion (a → b in Figure 9). Then an acid is necessary for the formation of the stable intermediate HET from the enamine or α-carbanion (IV) which is generated immediately

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after decarboxylation (c → d). Finally, a base is required to abstract a proton from the hydroxyl group of HET in the final step of releasing the product and the ylide (d → a).

Schellenberger originally proposed that the 4′-amino group serves as an intramolecular based and acid alternatively through the formation of an amino–ammonium pair in the V form.[3a] Sable et al. did not support this model which involves an N^+-(ammonium)–H···O(2α) hydrogen bond, based on the chemical evidence that the 4′-amino group in thiamin is relatively unreactive.[1b] They implicitly proposed an active V model of HET in which the N–H···O(2α) hydrogen bond assists the initial alignment of pyruvate to the thiazolium ylide. However, their V model differs from ours in the conformation of the C(2) substituent. Based on the absolute requirement of the N(1′) atom for binding and catalytic activity, Schellenberger revised his original proposal and presented the following proton relay mechanism:[3b,d] in the adduct formation step, an H atom is transferred to the keto O(2α) of pyruvate from the 4′-amino group which acts as an acid and becomes an imino group with the formation of an N(imino)···H–O(2α) hydrogen bond; in the product release step , the hydroxyl H atom is transferred to the imino group which thereby acts as a base and becomes an amino group. N(1′) is depicted to play an essential role by being unprotonated in the amino state and protonated in the imino state. In the three protein crystal structures at current resolutions, N(1′) is hydrogen-bonded to a glutamic O atom, and there are no acidic or basic amino acids around C(2).[5-7] In fact, earlier evidence that the 4′-amino group acts as a weak acid when N(1′) is protonated had been demonstrated in extensive model works by Jordan.[49]

Apparently, Schellenberger's mechanism seems consistent with the chemical and X-ray results. However, careful consideration indicates that an extra acid other than the 4′-amino group is still necessary for the formation of stable HET from the enamine or α-carbanion in the third step (c → d). Accordingly, the 4′-amino group alone cannot satisfy the roles of all the acids and bases necessary in the catalytic cycle. This point leads us to propose an alternative mechanism which is also consistent with the inhibitory property of the potent inhibitor oxythiamin. It has been known that PDC catalyzes the formation of the oxythiamin–pyruvate adduct and its decarboxylation, but the enzyme does not release the product acetaldehyde. The reaction terminates in the formation of an enzyme-bound dead-end product 2-(α-hydroxyethyl)oxythiamin.[3a] This inhibitory property of oxythiamin suggests that the 4′-amino group may not be as essential prior to the product release step as Schellenberger had believed. It is very likely that oxythiamin binds to the enzyme in the V form and proceeds to catalyze the adduct formation and decarboxylation in the same V(+ −)B conformation as thiamin does. The similarity in conformational properties of the C(2) substitution products of thiamin and oxythiamin is known from the crystal structures of 2-(α-hydroxybenzyl)thiamin[10] and 2-(α-hydroxybenzyl)oxythiamin.[12] As readily as the N–H···O(2α) hydrogen bond can be formed in HET in the V form, the O(4′α)···H–O(2α) hydrogen bond can just as easily be formed in 2-(α-hydroxyethyl)oxythiamin in the V form. In the latter case, the hydroxyl H atom would become inert toward a base which would make 2-(α-hydroxyethyl)oxythiamin a dead-end product, and thus acetaldehyde would not be released. If this *reversed* hydrogen bond is a major factor for the inhibitory property of oxythiamin, the comparable N(imino)···H–O(2α) hydrogen bond in Schellenberger's model may also be an inhibitory rather than a catalytic factor. Therefore, one still cannot exclude other possibilities than that the 4′-amino group is the sole acid–base function. It may be possible that a conformational change upon binding the substrate may bring an acidic or basic residue near the active center, even though there is apparently no acidic or basic residue around the C(2) active center in the proteins. In this regard it is noteworthy that only the coenzyme but not the substrate occurs in the three known protein structures. It may also be possible that a water molecule can act as the required acid or base, at least, in some stage of catalysis. Further study is required to elaborate this point in order to understand thiamin catalysis more completely.

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Supplementary Material Available: Extended version of Table I including the torsion angles $\phi_{5\alpha}$ and $\phi_{5\beta}$ and the full list of references (5 pages). This material is contained in many libraries on microfiche, immediately follows this article in the microfilm version of the journal, and can be ordered from the ACS; see any current masthead page for ordering information.

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