The polar fraction, 146 mg (92%), gave the required (*S*)-(+)-hydroxyaldehyde **8a** as a solid: mp 48-52 °C; $[\alpha]^{25}_{Hg}$ +67.54 ± 0.20° (*c* 0.86, $CHCl_3$); IR (0.03 M solution in CCl_4) 3585 (sharp), 3460 (br), 2900 (m), 1670, 1600, 1476, 1400, 1380, 1220, 1190, 1150, 1070, 1050, and 990-880 cm^{-1}; ^1H NMR 1.20 (s, 3 H), 1.27 (s, 3 H), 1.47 (s, 3 He, 1.53 (s, 3 H), 1.50-2.00 (m, 5 H), 4.12 (m, 1 H), 6.02 (d, J = 7.68, 1 H), and 10.42 (d, J = 8.05, 1 H); UV (*c* 1.23 × 10^{-4}, 1.23 × 10^{-2}, cyclohexane) λ_{390} ε 8, λ_{272} ε 24, λ_{354} ε 43, λ_{341} ε 46, λ_{330} ε 41, λ_{320} ε 33, and λ_{242} ε 3+ 700; CD (*c* 1.23 × 10^{-3}, 1.23 × 10^{-2}, cyclohexane) $\Delta\epsilon_{392}$ -0.07, $\Delta\epsilon_{373}$ -0.26, $\Delta\epsilon_{357}$ -0.41, $\Delta\epsilon_{342}$ -0.39, $\Delta\epsilon_{330}$ -0.27, $\Delta\epsilon_{320}$ -0.14, $\Delta\epsilon_{242}$ +6.15, and $\Delta\epsilon_{204}$ -2.33.

Anal. Calcd for $C_{12}H_{20}O_2$: C, 73.46; H, 10.20. Found: C, 73.03; H, 10.20.

X-ray Data for 4-Hydroxy-2,2,6,6-tetramethylcyclohexylideneacetic Acid. Single crystals of $C_{12}H_{20}O_3$ were grown by slow evaporation from acetone solvent. The crystals were orthorhombic, space group $P2_12_12_1$ with a = 7.437 (1) Å, b = 10.122 (2) Å, c = 15.845 (5) Å and d_{calcd} = 1.260 g cm^{-3} for Z = 4 (M_r = 226.32). The intensity data were measured on a CAD4 Enraf Nonius diffractometer (Mo radiation, monochromated, θ-2θ scans). The size of the crystal used for collection was approximately 0.3 × 0.3 × 0.3 mm^3. No absorption correction was necessary (μ = 0.818). A total of 1252 reflections was measured for $\theta \leq 25.0$, of which 944 were considered to be observed [$I \geq 2\sigma(I)$]. The structure was solved by direct methods using MULTAN 78 (Main, Peter MULTAN 78. *A System of Computer Programs for the Solution of Crystal Structures from X-ray Diffraction Data*; Department of Physics, University of York: York, England.) and refined by full-matrix least-squares methods.

In the final refinement, anisotropic thermal parameters were used for non-hydrogen atoms. Most of the hydrogen atoms, with the exception of some of the methyl hydrogen atoms, were measured from a difference Fourier map; the remaining hydrogen atom parameters were calculated assuming idealized geometry. Hydrogen atom contributions were included in the structure factor calculations, but their parameters were not refined. The final discrepancy indexes were R = 5.9 and R_w = 6.5 for the 944 observed reflections. The final difference Fourier map was essentially featureless; the highest residual peaks were in the vicinity of the carboxyl group and had densities of 0.3 e A^{-3}.

Acknowledgment. We thank Dr. M. Zoda for his early exploratory experiments and Dr. M. Duraisamy for many helpful discussions.

Supplementary Material Available: Crystal data, interatomic distances, selected bond angles, selected torsional angles, table of positional and thermal parameters and their estimated standard deviations, and structure factors (F_{obsd} and F_{calcd}) for $C_{12}H_{20}O_3$ (10 pages). Ordering information is given on any current masthead page.

Enzymic Carboxyl Transfer from *N*-Carboxybiotin. A Molecular Orbital Evaluation of Conformational Effects in Promoting Reactivity

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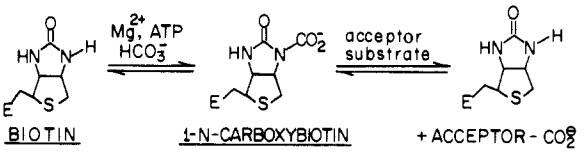
Abstract: On the basis of analysis of conformational energetics and enzymatic reactivity patterns, it is shown that the activation of *N*-carboxybiotin toward carboxyl transfer can be initiated by rotation of the carboxyl group out of the plane of the urea ring of *N*-carboxybiotin. This rotation provides proper polarization and bond weakening as expected from resonance models. It also provides a correct alignment for bond formation between the carbanion derived from the substrate and the carboxyl group of *N*-carboxybiotin. The proposal is supported by molecular orbital calculations on ground-state and transition-state models. These calculations also indicate that puckering of the imidazolidone ring does not lower activation barriers. The activation energy calculated for bond rotation is consistent with measured barriers in related systems. The mechanism accounts for the low nonenzymic activity of *N*-carboxylated ureas, the source of enzymic activation, and the recently reported "triggering" of *N*-carboxybiotin by substrate analogues.

The pioneering work of Lynen[1] established the intermediacy of carboxylated biotin in enzymic carboxyl-transfer reactions. However, the mechanism of transfer has still not been elucidated. The site on biotin that is carboxylated was proposed to be one of the nitrogen atoms or the urea oxygen atom. On the basis of model studies and reactivity analysis, Bruice and Hegarty[2] advocated carboxylation at oxygen. However, later enzymic work by Lane and co-workers[3] has led to the general acceptance of the much less reactive *N*-carboxylated species. Of course, the low reactivity of N-carboxylated ureas toward carboxyl transfer[4,5] makes *N*-carboxybiotin an ideal agent for the safe transfer of a carboxyl group between carboxyl-transfer sites (Scheme I), which is an important function of this enzymic intermediate. However, this lack of reactivity must be dramatically altered in the presence of the enzymic subunit responsible for carboxyl transfer, since, after an acceptor substrate binds, facile transfer of the carboxyl group occurs.[6]

Scheme I



The chemical basis for this change in reactivity is unresolved. Mechanisms that have been proposed rely on enolization of the urea moiety to promote N1-C3 bond cleavage (Scheme IIa-c).[7-10]

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(2) Bruice, T. C.; Hegarty, A. F. *Proc. Natl. Acad. Sci. U.S.A.* **1970**, *65*, 805.
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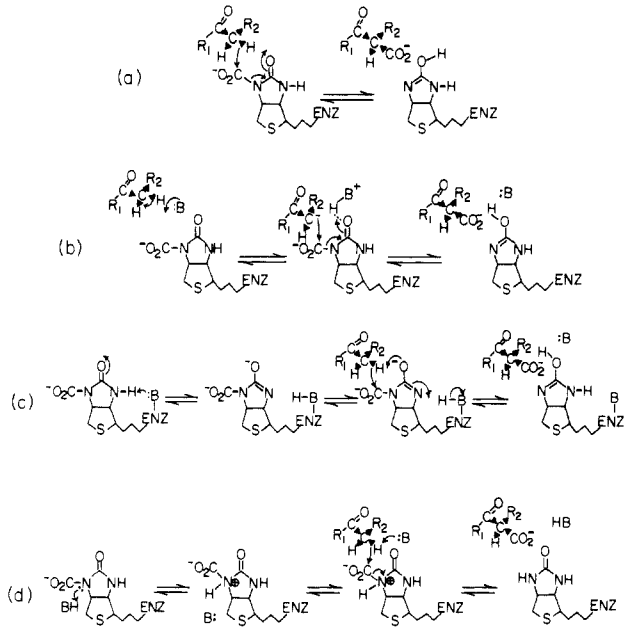
0002-7863/86/1508-2699$01.50/0

Scheme II



In mechanisms (a) and (b), if O1–H bond formation precedes C–C bond formation, then bonding to O1 is seen as the activating process; if C–C bond formation precedes O1–H bond formation, then attack on the carboxyl group is the activating process. Recent work by Wallace on pyruvate carboxylase would seem to rule out both these mechanisms, since nonreacting substrate analogues are shown to activate *N*-carboxybiotin toward carboxyl transfer (in this case CO_2 is transferred to water).[11] These substrate analogues (glyoxalate, oxamate) do not possess a labile proton for transfer to O1 and cannot form carbanions for attack on carboxylate.

Mechanism (c), proposed in response to these results, involves ionization of the urea group of *N*-carboxybiotin. Since this group is a very weak acid, formation of the conjugate base will be energetically unfavorable (cf. *N*-methylurea; $pK_a = 18.3$[12]). Its formation also requires an enzyme base at the active site for which there is no evidence. An alternative mechanism (Scheme IId) is consistent with Wallace's results but again requires a high-energy species that results from the thermodynamically unfavorable protonation at N3[13] [$K_a(O1)/K_a(N3)$ is estimated as $>10^{7}$[14]]. If either the conjugate base or conjugate acid is to form, special stabilization at the active site must be involved, which itself must require energy.

Examination of the resonance contributors to *N*-carboxybiotin, combined with structural analysis based on available X-ray data, led us to consider that a mode of activation may exist that utilizes a simple conformational change in the ground state. This mode is rotation of the carboxyl group out of the plane of the urea ring.

X-ray analysis shows the *N*-carboxyurea moiety of *N*-carboxybiotin to be planar and hence fully conjugated.[15] The rigid, fused bicyclic ring system of *N*-carboxybiotin allows little conformational flexibility, which might perturb this conjugation. Single-bond rotations that can affect conjugation are limited to puckering of the urea ring and rotation of the carboxyl group. If one considers the most likely resonance contributors to *N*-carboxybiotin (Figure 1), it can be seen that rotation of the carboxyl group out of the plane will result in loss of one major

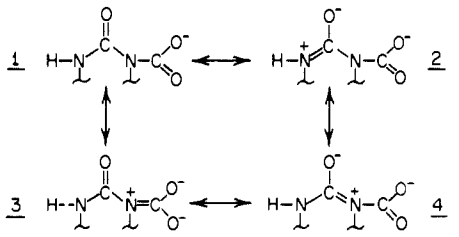


Figure 1. Major resonance contributors to the *N*-carboxyurea moiety of *N*-carboxybiotin.

Table I. Optimized Bond Lengths and Angles for Structure II

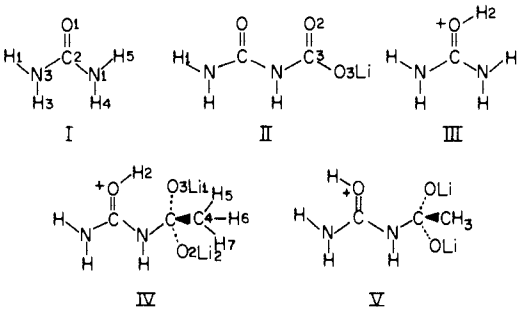
bond	length, Å	bond angle	angle, deg	dihedral angle	angle, deg
C2–O1	1.218	N1–C2–O1	124.8	C3–N1–C2–O1	−0.2
C2–N1	1.430	N3–C2–O1	123.4	H1–N3–C2–N1	180.0
C2–N3	1.413	H1–N3–C2	118.8	O3–C3–N1–C2	180.0
N3–N1	1.013	H4–N3–C2	122.9	Li–O3–C3–N1	180.0
N3–H3	1.012	H3–N1–C2	118.8		
N1–H4	1.018	C3–N1–C2	124.3		
C3–N1	1.440	O3–C3–N1	111.3		
C3–O3	1.354	O2–C3–N1	124.1		
C3–O2	1.218	Li–O3–C3	185.0		
O3–Li	1.472				

contributor (**3**). The loss of this contributor should weaken the N1–C3 bond and increase polarization of the molecule. These changes promote reactivity. This single-bond rotation thus constitutes a potential activation process for carboxyl transfer compatible with all available data. We examined the feasibility of such a conformational activation mechanism employing molecular orbital calculations on model structures (a similar approach has been applied by Jean and Lehn[16] to activation of CO_2 toward nucleophilic attack).

Calculations

All optimized structures were partially constrained to mimic the planar urea ring of biotin: H3, N3, C2, O1, and N1 were maintained in the same plane.

By use of the program MONSTERGAUSS[17] with an STO-3G minimal basis set,[18] models of biotin (I), *N*-carboxybiotin (II), and proton-



ated urea (III) were geometry optimized. The tetrahedral transition-state structure (IV) represents attack of a carbanion (in this case methyl) on the carboxyl group of *N*-carboxyurea. In accord with proposed mechanisms for carboxyl transfer to *N*-carboxybiotin, the urea oxygen is protonated. The proton is directed toward N1, accommodating a direct transfer mechanism. In order to achieve the best theoretically optimized geometry, the minimum number of constraints was employed (6 of the 45 geometric parameters constrained; methyl protons set equivalent; all Li–O lengths and Li–O–C3 angles set equal). The urea ring (H3–N3–C2–N1–H4) was maintained planar, and the dihedral angles of the methyl protons were set at 120°. Geometry optimization of IV progressed to a minimum gradient length of 7.5×10^{-3} without convergence. Optimization was aborted after a further 19 iterations, since gross structural changes were occurring, involving bonding between H2 and O2 and Li2 and O3.

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Table II. Partially Optimized Bond Lengths and Angles for Structure V

bond	length, Å	bond angle	angle, deg	dihedral angle	angle, deg
C2–O1	1.348	N1–C2–O1	124.9	H1–N3–C2–N1	180.0
C2–N1	1.350	N3–C2–O1	123.2	C3–N1–C2–O1	0.0
C2–N3	1.344	H1–N3–C2	121.6		
N3–H1	1.022	H4–N3–C2	120.9		
N3–H3	1.022	H3–N1–C2	119.5		
N1–H4	1.022	H2–O1–C2	109.4		
O1–H2	0.991	C2–N1–C3	120.6		
C3–N1	1.539	C4–C3–N1	109.4		
C3–C4	1.581	C3–C4–H	110.1		
C4–H	1.086	O3–C3–N1	105.3		
Li–O	1.526	O2–C3–N1	106.9		
C3–O3	1.422	Li–O3–C3	200.9		
C3–O2	1.422				

As a result of the nonconvergence of structure IV, a modified model of the transition state (V) was constructed to be used in conformational analysis. The geometry of V was derived from fully optimized protonated urea (III) and from the minimum gradient geometry of IV. Both lithoxy ligands were set to be structurally identical, consistent with expectations for the true transition state. Both O–Li bonds were directed away from the urea moiety so as not to interfere in the surface scans. H2 was directed toward H1 to prevent the interactions with O2 and O3, which had occurred in geometry optimization of IV. The bond distances and bond lengths derived for structures II and V are given in Tables I and II, respectively.

Structural changes were carried out on II and V, corresponding to conformational changes in the *N*-carboxybiotin molecule. In order to correlate activation of *N*-carboxybiotin with conformation, these structural changes were carried out in increments and the molecular energy and Mulliken population analysis calculated for each structure. The changes in the model systems correspond to (i) puckering of the ring system, (ii) pyramidalization at the ureido nitrogens (i.e., increase in sp^3 character), and (iii) rotation of the carboxyl group. The structural changes that correspond to these conformational motions are, respectively, (i) rotation about C2–N1 (θ in Figure 4) and/or C3–N1, (ii) movement of H1 or C3 out of the urea plane, and (iii) rotation about N1–C3 (ϕ in Figures 2, 3, and 4).

Two approaches were used to analyze the conformational activation of *N*-carboxybiotin. First, we examined the mechanism of enzymic decarboxylation of *N*-carboxybiotin in terms of the stereoelectronic properties of the ground state. In order to verify our predictions based on resonance structure analysis, one must analyze the charge density at each atom as a function of carboxyl group rotation (although the overlap population of the N1–C3 bond would indicate the effect of conformation on N1–C3 bond strength, the unreliability of Mulliken overlap population analysis in such calculations is well documented[19]). For a more detailed analysis of the effect of puckering, CO_2 rotation, and N-pyramidalization on ground-state *N*-carboxybiotin, we concentrate on the factors that should promote carboxyl-group transfer: (i) increased electrophilicity at the C3 position; (ii) increased basicity at the ureido oxygen O1 (assisting proton abstraction from the substrate or BH); (iii) increased overlap of a lone pair on N1 with the urea π system (assisting departure of the carboxylated substrate through stabilization of the incipient negative charge by the urea π system). Two nitrogen lone pairs are available for overlap: the π-symmetrized lone pair and the incipient lone pair formed on C3–N1 bond cleavage.

These factors were evaluated as a function of conformation in the model system by following indicators in the electronic distribution across II: (i) charge density at C3; (ii) charge density at O1; (iii) the degree of overlap between an N1 lone pair and the π system (given by the dihedral angle C3–N1–C2–O1, optimum overlap occurring at multiples of $\pi/2$ rad).

Second, we considered cross sections of the Born–Oppenhiemer energy surface corresponding to conformational changes. We applied the principle of least nuclear motion[20] and assumed that the ground state is sufficiently flexible that the most favorable conformation for decarboxylation is that which leads to the lowest activation energy between reactant and transition state along an accessible energetic pathway. This conformation will probably differ from the conformational minimum. Structure V approximates the expected transition state: the energy difference between equivalent conformers of II and V approximates the activation energy (eq 1).

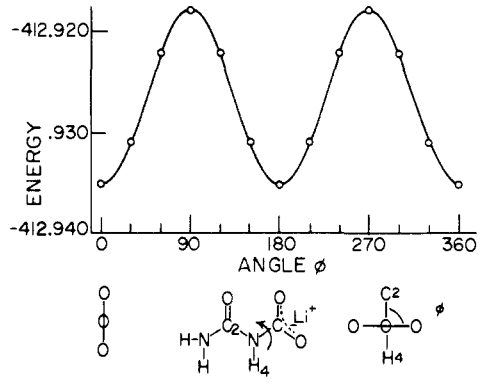

Figure 2. Effect of rotation of the carboxyl group of structure II upon molecular energy in Hartree unity. Minimum energy corresponds to coplanar conformation; maximum occurs with carboxyl perpendicular to plane of urea. Newman projection looks down the C3–N1 bond from C3.

$$E_a = E(\mathrm{V}) - [E(\mathrm{II}) + E(CH_4) + E(Li^+)] \quad (1)$$

A value of −47.1 hartrees was taken for $E(CH_4) + E(Li^+)$ in calculation of activation energies.[21] These values are required since the transition-state model (V) is formed from addition of CH_4 and Li^+ to the ground state (II).

The inclusion of lithium ions was necessary to balance the negative charges on the oxyanions. Negative charges can cause problems in closed-shell STO-3G calculations. For the *N*-carboxybiotin model (II), pairs of conformations were averaged so as to make O2 and O3 necessarily equivalent.

Results

Ground-State *N*-Carboxybiotin Analysis. The effect of carboxy group rotation on the energy of the *N*-carboxybiotin model (II) can be seen in Figure 2. The planar structure is the most stable, the barrier to carboxyl group rotation being approximately 11 kcal mol^{-1}. This value would be minimized further on geometry optimization of the maximum energy structure ($\phi = 90°$). An 11 kcal mol^{-1} energy barrier to rotation is at the lower end of the range for substituted amides (experimentally determined examples: *N,N*-dimethylacetamide, $\Delta G_c^{\ddagger} = 18.9$ kcal mol^{-1};[24] methyl *N,N*-diethylcarbamate, $\Delta G_c^{\ddagger} = 14.4$ kcal mol^{-1}[25]). This is as expected since cross conjugation with the urea group in *N*-carboxyurea will further weaken the C–N bond relative to carbamate. The value is nevertheless substantial.

The result is consistent with the planar structure of the ureido ring of biotin, which has been reported by DeTitta et al.[26] The geometry of the optimized model (II) is also consistent with X-ray data for *N*-(methoxycarbonyl)biotin methyl ester.[15] Changes in the geometry of the *N*-carboxybiotin model (II) relative to urea mirror those in *N*-(methoxycarbonyl)biotin relative to biotin (Table III). The presence of the carboxyl group increases the C2–N1 bond length relative to C2–N3. This is due to a shift of the delocalized lone pair associated with N1 from the C2–N1 π bond into the C3–N1 π bond. In turn, this leads to significant dou-

(19) (a) Fliszar, S. *Charge Distribution and Chemical Effects*; Springer-Verlag: New York, 1983. (b) Streitweiser, A., Jr.; Collins, J. B.; McKelvey, J. M.; Grier, D.; Sender, J.; Toczko, A. G. *Proc. Natl. Acad. Sci. U.S.A.* **1979**, *76*, 2499 and references cited therein. It should be noted that N1–C3 overlap population does fall to a minimum on rotating the carboxyl group through 90° and increases with puckering. The variation is very modest (4 millielectron) possibly since the N1–C3 bond length is kept constant.

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(21) This is a reasonable value based on those contained in the literature: e.g., $E_{HF}(CH_4)$/hartree = −39.72686[22] and $E_{HF}(Li^+)$/hartree = −7.13545[22] and −7.4780.[23] For comparison, E_{HF}(geometry-optimized model II)/hartree = −412.93005.

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Table III[a]

		bond lengths, Å			
		C2–O1	C2–N3	C2–N1	C3–N1
STO-3G	biotin[b]	1.222	1.412	1.412	
	NCB[c]	1.218	1.413	1.430	1.440
X-ray	Biotin	1.249	1.351	1.322	
	NCB	1.207	1.345	1.405	1.392

[a] Employing the numbering system used in this paper. NCB is *N*-carboxybiotin. [b] Urea. [c] Model 11.

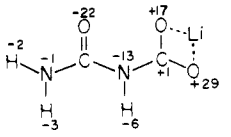

Figure 3. Change in electron density at each atom of structure 11 on rotating the carboxyl group through 90° from the coplanar conformation (i.e., $\phi = 0°$ to $\phi = 90°$). Positive values indicate increase in positive charge, given in millielectrons. There is no change at the lithium atom. The total change across the system is 49 millielectrons.

ble-bond character in the C3–N1 bond.[27,28] Thus, molecular orbital calculations confirm the evidence from X-ray data: resonance structure **3** does represent a significant feature of the ground state of *N*-carboxybiotin.

The change in charge distribution across model II on rotating the carboxyl group perpendicular to the urea ($\phi = 0°$ to $\phi = 90°$) is shown in Figure 3. The change in charge density at each atom in the perpendicular conformation ($\phi = 60°$) relative to the planar conformation ($\phi = 0°$) is given in millielectrons (where positive values indicate increases in positive charge). Again, all values are consistent with the prediction based on resonance structures: structure **3** does make a significant contribution, and this contribution is removed on rotation about the N1–C3 single bond. The elimination of π overlap between N1 and C3 weakens the N1–C3 bond.

Carboxyl rotation results in increased electron density in the urea moiety due to withdrawal of density from the N1–C3 π bond. This is compensated by movement of electrons from the carboxylate oxygens. The decreased electron density at the carboxylate oxygen atoms promotes carboxyl transfer as well as withdrawal of electron density into the urea group. Increased electron density at the urea oxygen makes this site more readily protonated. Protonation of the urea oxygen is necessary for enol formation and is a required step whether the carboxylate group is transferred to water or to the substrate. The calculated changes also support the significance of coplanarity in the stability of *N*-carboxyureas. Rotation of the carboxyl group is a simple and direct route to promote carboxyl transfer as well as enol urea formation.

It may be possible that only a small rotation of the carboxyl group out of the plane (i.e., $\phi < 90°$) is adequate to reduce N1–C3 conjugation sufficiently to activate *N*-carboxybiotin to carboxyl transfer. On the other hand, an alternative conformational change might result in activation. To analyze the significance of these possibilities, we examined the three stereoelectronic factors outlined above in terms of more detailed conformational changes.

Examination of molecular models shows puckering of the urea ring of *N*-carboxybiotin may be approximated by single-bond rotations: rotation about N1–C2 providing one puckering mode and rotation about N3–C2 providing a second puckering mode. Of the two puckering modes and N-pyramidalization, only the N1–C3 puckering mode yields a positive effect on the reactivity of the *N*-carboxybiotin model. The combined effects of this puckering mode with carboxyl group rotation are illustrated in Figure 4.

From Figure 4 it can be seen that rotation of the carboxyl group alone allows maximum negative charge on O1 (A). The variation

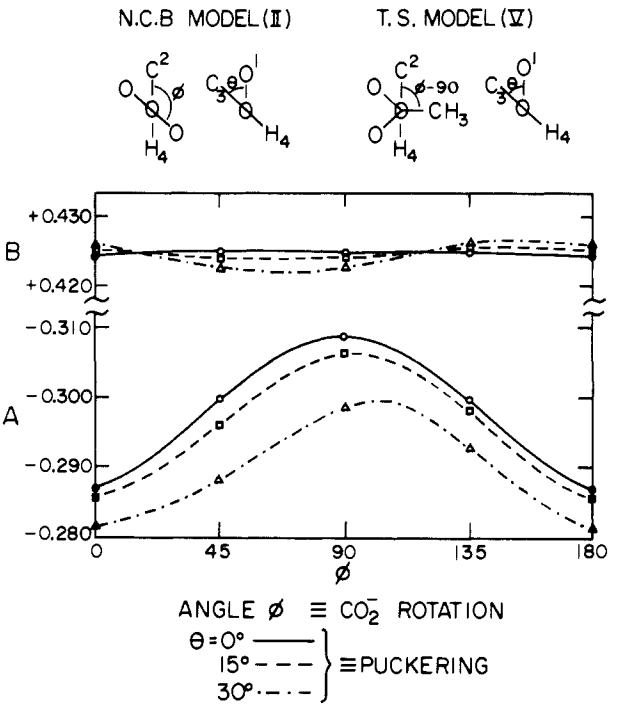

Figure 4. Simulation of carboxyl group rotation combined with ureido-ring puckering (puckering is equivalent to rotation about C2–N1, given by θ). Effect of conformational change to structure 11 on charge distribution (see Calculations section): (A) charge density at the urea oxygen (O1); (B) charge density at the carboxyl carbon (C3). Newman projections shown view the C3–N1 bond from C3 to illustrate ϕ and view the N1–C2 bond from N1 to illustrate θ. Note that ring puckering destroys the symmetry observed on carboxyl group rotation about the planar urea ring.

in charge density at C3 (B) is negligible. Additional conformational change through puckering ($\theta = 15°$ or 30°) yields only a very small increase in electrophilicity at C3 (A) ($\phi < 20°$, $\phi > 120°$). π systems overlap does not vary with carboxyl group rotation but is reduced considerably by puckering. As given by the C3–N1–C2–O1 dihedral angle, π overlap decreases from the optimum value of $\pi/2$ rad at $\theta = 0°$ to a value of $\pi/3$ at $\theta = 30°$ (minimum overlap is indicated by a value of $\pi/4$ rad). In terms of these three stereoelectronic factors, any degree of rotation out of the plane increases reactivity, a maximum occurring when the plane of the carboxyl group is perpendicular to the plane of the urea ring. At $\phi = 90°$, O1 carries maximum negative charge, indicating the largest degree of polarization in the urea and largest withdrawal of electron density from the N1–C3 bond.

Molecular orbital studies have been used to study activation of carbonyl species toward nucleophilic attack at carbonyl carbon.[16,27] These studies indicate that activation correlates with the energy of the π^* orbital (C*) of the carbonyl species rather than the electron density at the carbonyl carbon. In our system, the electron density at the carbonyl carbon varies only slightly, since density drawn from C3 into the urea moiety is compensated by withdrawal of density from the carboxylate oxygens into C3. However, the eigenvalue of the lowest unoccupied molecular orbital, with π^* character associated with the carboxyl moiety, does decrease with rotation of the carboxyl group to a minimum value at $\phi = 90°$.

Activation Energy Analysis. Figure 5 illustrates variation in activation energy with conformation by correlating conformational changes in the *N*-carboxybiotin ground state with the tetrahedral transition state. The values of activation energy (E_a) are inaccurate in an absolute sense since (i) the transition-state structure (V) is not fully geometry-optimized, (ii) the values are dependent on the number selected for $E_{HF}(Li^+) + E_{HF}(CH_4)$, and (iii) differential solvation energies for the ground and transition states at the active site are neglected in ab initio calculations. However, since all these factors are internally compensating with respect to conformational changes, the trends in activation energy with

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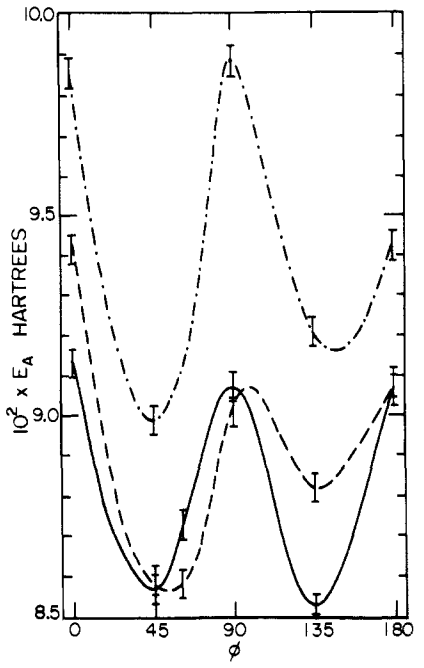

Figure 5. Simulation of carboxyl group rotation combined with ureido-ring puckering: effect on activation energy for CO_2 transfer. Activation energy (E_a) is given by 1. For legend, see Figure 4. N.B. A small difference in the N1–C3–O bond angles (1.5°, see Table II) results in the curve for $\theta = 0°$ being slightly dissymmetric about $\phi = 90°$.

conformation are reliable and significant.

It can be seen that there is no real advantage to be gained from puckering of the urea ring through any angle (Figure 5). In fact, significant out-of-plane bending causes an increased barrier to reaction in spite of an increase in the enthalpy of the reactant.

Rotation of the carboxyl group does yield a net decrease in activation energy. The minimum E_a values occur for a clinal conformation (at $\phi = -45°$ and $+45°$, the plane of the carboxyl group is midway between perpendicular and periplanar relative to the plane of the urea moiety). It is possible that the local maximum observed when the carboxyl group is perpendicular to the urea moiety ($\phi = 90°$) is artificial, since V is not geometry optimized (steric repulsion between the methyl group and the lone pair on oxygen is probably responsible for this maximum[29]). The geometric parameters used for this part of the structure of V are derived from the partially geometry-optimized (i.e., minimum gradient length) parameters for IV where $\phi = +8°$. Geometry optimization of IV or V, in which ϕ is constrained at 90°, would minimize this steric repulsion and hence lower the activation energy for the structure in which the plane of the carboxyl group is perpendicular to the plane of the urea. Of course, $\phi = 45°$ may correspond to a true minimum in the energy hypersurface along which carboxyl transfer occurs.

These results indicate that reaction of the carboxyl group out of the plane of the urea ring will activate *N*-carboxybiotin toward carboxyl transfer. A clinal orientation is favored in terms of activation energies, which assume a tetrahedral transition state, whereas a perpendicular orientation is predicted from examination of the stereoelectronic characteristics of the ground state.

Discussion

Significance of Coplanarity in *N*-Carboxybiotin. In terms of these molecular orbital calculations, any puckering of the ureido ring of *N*-carboxybiotin will reduce reactivity toward carboxyl transfer. Puckering by C2–N1 rotation also converts the urea functionality into an amino-substituted amide. This reduces the basicity of the ureido oxygen (the pK_a of the conjugate acid of *N*-methylurea is 0.9;[30] the pK_a of the conjugate acid of acetamide is −0.9[31]). Since the ureido oxygen must become protonated during reaction, such a change in basicity is counterproductive. It is therefore likely that the planar ureido ring in *N*-carboxybiotin is also necessary for maintenance of the basicity of the ureido oxygen, required for transfer of the carboxyl group. Interestingly, a number of biotin analogues that do not have a fusion to a tetrahydrothiophene ring show puckering in the ureido ring,[32,33] suggesting that the function of the sulfur atom may be structural in maintaining the planarity of the urea ring.

Nature of the Transition State. Both stereoelectronic and activation energy analyses of the molecular orbital calculations show carboxyl group rotation to be an activating process toward carboxyl transfer. Results based on activation energy analysis are premised on V being a reasonable representation of the true transition state. This premise is supported by the tetrahedral transition state proposed by Caplow[4] for the decarboxylation of *N*-carboxyimidazolidone esters. The structure of V is also consistent with experimental and ab initio studies on the stereochemistry of the tetrahedral adduct resulting from nucleophilic addition to carbonyl carbon.[34,35] Although it is possible that V represents an intermediate rather than a transition state in decarboxylation, the activation energy approach is still valid, since this intermediate would be expected to be a high-energy species resembling the transition state. Since the nature of the ground state is known, whereas that of the transition state is approximated, the results from stereoelectronic analysis must be considered more generally applicable than those from the activation energy approach.

Carboxyl Transfer via CO_2 Formation. Sauers and Jencks have suggested that carboxyl transfer from *N*-carboxybiotin proceeds via free CO_2.[36] This is clearly inconsistent with an activation energy approach based on a tetrahedral transition state, but such a mechanism will also benefit from carboxyl group rotation. The stereoelectronic approach remains valid if free CO_2 is involved in carboxyl transfer, since the ground state of *N*-carboxybiotin is common to both pathways involving a tetrahedral intermediate or free CO_2. Weakening of the N1–C3 bond, withdrawal of charge from the carboxylate oxygens into the carboxyl carbon, and enolization of the urea moiety will all promote carboxyl transfer by mechanisms involving generation of CO_2. These are exactly the changes caused by rotation of the carboxyl group out of the urea plane.

The charge distribution of the carboxylate group of the *N*-carboxybiotin model (II) can be compared with that of CO_2. The charge density at carbon and oxygen for CO_2 is +0.466 and −0.233 respectively.[16] This compares with +0.424 and −0.303 (average of O2 and O3) for *N*-carboxybiotin in its stable planar conformation. Rotation of the carboxyl group of *N*-carboxybiotin through 90° changes the charge density at carbon and oxygen to +0.425 and −0.279 respectively, thus promoting CO_2 formation.

Stereochemistry of Carboxyl Transfer. The direct transfer mechanism proposed by Kuo and Rose (Scheme IIa) is inconsistent with transfer from planar *N*-carboxybiotin in terms of what is known about the stereochemistry of the reaction: (i) transfer of the carboxyl group from *N*-carboxybiotin has been demonstrated to proceed with retention of configuration at the substrate carbon,[37] (ii) attack of the nucleophile (substrate carbanion) on a carbonyl species (carboxyl of *N*-carboxybiotin) must occur in a plane perpendicular to the carbonyl group;[34,35] (iii) the optimum geometry for proton transfer between two heavy atoms (O1 and C4 in this case) occurs when the three atoms are collinear.[38] For

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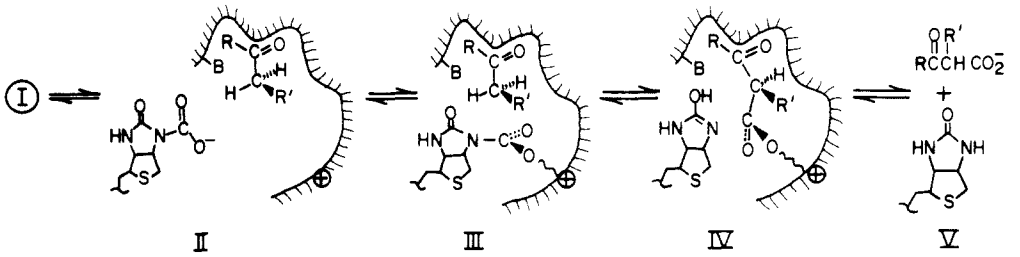

Figure 6. Enzymatic carboxyl group transfer via *N*-carboxybiotin: (I) N-carboxylation of biotin at first subsite of carboxylase; (II) transport phase, *N*-carboxybiotin in planar, unreactive conformation and substrate at second substite; (III) activation of *N*-carboxybiotin at second site by rotation of carboxyl group through 90°; (IV) transfer of CO_2 to substrate with retention of configuration; (V) dissociation of carboxylated substrate and regeneration of biotin through tautomerization.

direct transfer from planar *N*-carboxybiotin, the H bond between O1 and substrate carbanion would necessarily be long and severely bent. With the carboxyl group perpendicular to the urea plane, all species are alligned for a direct transfer mechanism. For a stepwise mechanism (with or without the intermediacy of an enzymic base (Scheme IIa,b), the stereochemical restrictions are less rigid, but in terms of the principle of least motion, transfer from perpendicular *N*-carboxybiotin would again be favored.

Without detailed knowledge of the energetics of the carboxy-transfer process, it is impossible to predict whether rotation of the carboxyl group through a full 90° is necessary to activate *N*-carboxybiotin. For example, it may be that rotation through a smaller angle (which will require less energy) is sufficient to overcome the barrier to carboxyl transfer. Higher level molecular orbital calculations may provide more information.

Conclusion

It has been previously demonstrated that when an acceptor substrate is bound at the active site of the second subunit of pyruvate carboxylase, *N*-carboxybiotin is activated such that facile transfer of CO_2 to substrate occurs.[6] In the presence of non-reacting substrates, transfer of CO_2 is to a water molecule.[7] One must propose a chemically reasonable mechanism in which binding energy is utilized to activate the molecule to carboxyl group transfer.[39]

We propose that rotation of the carboxyl group of *N*-carboxybiotin out of the plane of the urea ring is a readily accessible mode of activation that promotes carboxyl transfer via the previously proposed stepwise or concerted mechanisms[7-10] (Scheme IIa,b) or via free CO_2.

Figure 6 illustrates a possible chain of events. Translocation of *N*-carboxybiotin to the second subsite is initiated as a result of substrate binding at that site. Interaction of *N*-carboxybiotin with a group at the second subsite of the protein causes rotation of the carboxyl group, activating the molecule towards transfer of CO_2 to substrate. The active-site residue represented as coordinating with the carboxyl group of *N*-carboxybiotin is shown as a cation. However, this binding interaction need not be electrostatic, since H bonding with a donor at the active site should readily cause carboxyl group rotation. It should be noted that a dissociative mechanism involving loss of CO_2 from *N*-carboxybiotin would be hindered by such electrostatic stabilization of the carboxylate at the active site. An associative mechanism involving formation of a tetrahedral adduct will be aided by such interaction.

The barrier to rotation is sufficient to maintain *N*-carboxybiotin in a stable planar conformation between the active sites (thus minimizing abortive hydrolytic cleavage), but not so large that transduction of enzymic binding energy cannot result in N1–C3 bond rotation. On the basis of data obtained by Green for binding of biotin analogues to avidin, one can anticipate the availability of up to 20 kcal mol^{-1} from binding of *N*-carboxybiotin.[40]

In summary, these molecular orbital calculations demonstrate that (i) the planar urea ring of *N*-carboxybiotin is necessary for carboxyl transfer; (ii) the coplanar arrangement of the carboxyl group relative to the urea ring confers stability on the molecule, preventing hydrolytic decarboxylation during the transfer phase between subunits; and (iii) rotation of the carboxyl group out of the plane of the urea ring activates *N*-carboxybiotin toward carboxyl group transfer.

Limitation and Extensions

It has already been stated that the activation energy approach is the weakest of the methods employed. To reemphasize this point it should be stressed that the structure used to model the transition state is not the structure of choice due to (i) problems, discussed above, with geometry optimization; (ii) the necessity for lithium ions; and (iii) the minimal representation of biotin and substrate due to the limitations of the algorithm. To obtain the true transition state a very large number of geometry optimizations would be necessary due to the large number of geometrical parameters that are likely to vary long the reaction coordinate. Further work to develop a more sophisticated approach is desirable. The methods used in the activation energy approach, including all its flaws, are discussed in detail. This should be taken into account in drawing conclusions based on the activation energy analysis.

We note that the particular problem we have dealt with is the duality of reactivity of *N*-carboxybiotin. Binding of *N*-carboxybiotin to the transcarboxylase subunit provides the energy to trigger rotation of the carboxyl group, which then permits facile reaction. Rotation of the carboxyl group presents a productive mode by which the enzyme may use binding energy to promote reaction. Efficient catalysis requires the usual additional factors that are part of enzymic reactions.

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