Exchange Characteristics of the Amide Protons of d-Biotin and Derivatives: Implications for the Mechanism of Biotin Enzymes and the Role of Sulfur in Biotin†

D. C. Fry, T. L. Fox, M. D. Lane, and A. S. Mildvan*

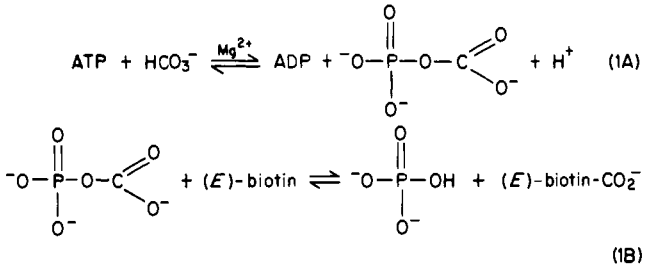
Contribution from the Department of Biological Chemistry, The Johns Hopkins University, School of Medicine, Baltimore, Maryland 21205. *Received April 24, 1985*

Abstract: The enzymatic carboxylation of d-biotin involves the replacement of the 1′-NH amide proton by 1′-COO^-. Transfer of saturation and T_1 measurements were used to determine the 1′- and 3′-NH proton exchange rates with water of free d-biotin (B), its methyl ester (BE), *O*-heterobiotin (OB), desthiobiotin (DB), and imidazolidone (IM). At pH 7.5 and 25 °C the exchange rate of the 1′-NH of B (58 s^{-1}) is similar to the carboxylation rates of enzyme-bound B (15 to 74 s^{-1}), indicating that deprotonation can precede carboxylation. Both H^+- and OH^--catalyzed exchange were faster at 1′-NH than at 3′-NH in B, BE, and OB but not in DB or IM, implying steric inhibition of exchange at 3′-NH by the side chain. At 3′-NH both H^+- and OH^--catalyzed exchange rates followed the order DB > IM > B > BE > OB, while at 1′-NH the order was B > BE > DB > IM > OB. While all other exchange rates were first order in $[H^+]$ or $[OH^-]$, that at 1′-NH of B and BE displayed an unprecedented 2nd order dependence on $[H^+]^2$, suggesting the protonation of both the carbonyl O and the 1′-N but not the 3′-N in the rate-limiting step. Simple first order H^+-catalyzed exchange with OB indicates that sulfur stabilizes the H_2B^{2+} and H_2BE^{2+} species, possibly by transannular bonding to the carbonyl carbon. H^+-catalyzed exchange at the 1′-NH of B had a lower enthalpy of activation (6 kcal/mol) than did the 3′-NH of B and the 1′- and 3′-NH of OB (9 kcal/mol). The rapid exchange of the 1′-NH and facile protonation of the amide carbonyl of B under mild conditions strongly support biotin carboxylation mechanisms involving enolization of the ureido system. The role of sulfur in biotin appears to be to increase the nucleophilicity of the 1′-N facilitating carboxylation at this position by transannular bonding to the carbonyl carbon of biotin.

Biotin serves as a prosthetic group on a number of enzymes which participate in carboxylation and carboxyl group transfer reactions.[1,2] These enzymes generally function in two steps.[3] In reaction 1, biotin is carboxylated at the 1′-N position via elec-

$$(E)\text{-biotin} + \text{ATP} + HCO_3^- \xrightleftharpoons{Mg^{2+}} (E)\text{-biotin-}CO_2^- + \text{ADP} + P_i \quad (1)$$

trophilic substitution for the 1′-NH proton. Reaction 1 is believed to involve a carboxyphosphate anhydride intermediate:[3] In



(1B)

reaction 2, the carboxylate group is transferred from carboxybiotin

$$(E)\text{-biotin-}CO_2^- + \text{acceptor} \rightleftharpoons (E)\text{-biotin} + \text{acceptor-}CO_2^- \quad (2)$$

to an acceptor substrate in exchange for a proton. The biotin enzyme transcarboxylase does not make use of reaction 1 but rather utilizes two consecutive reactions of type 2, initially removing a carboxyl group from one substrate, methylmalonyl CoA, and subsequently transferring it to another substrate, pyruvate.

Mechanisms which have been proposed for reaction 2 fall into two categories—concerted[4,5] and stepwise.[6-8] The concerted mechanism was suggested by the observations that carboxylation of propionyl CoA by propionyl-CoA carboxylase proceeds with retention of configuration[4] and that during this reaction there is no exchange of protons between substrate and solvent.[9] However, these considerations do not rule out a stepwise mechanism. In addition, the demonstration that transcarboxylase and propionyl-CoA carboxylase can eliminate HF from β-fluoropropionyl-CoA, without concomitant carboxylation of this substrate,[6] argues against a concerted reaction.

In this paper, we will consider the exchange characteristics of the amide protons at 1′-N and 3′-N of biotin and several of its derivatives. This provides information on the mechanism of the electrophilic substitution by which a carboxyl group replaces the amide proton at 1′-N both in reaction 1B and in the reverse of reaction 2. Possible mechanisms for the reverse of reaction 2 are outlined in Figure 1. These proposals also take account of the findings that pyruvate carboxylase and transcarboxylase contain Mn^{2+} and Zn^{2+}, respectively,[10,11] and the proximity of the metals on the enzymes to the bound substrates[12-14] suggesting a functional role for the metal atoms. In mechanisms A and A′ deprotonation at 1′-N of biotin precedes carboxylation. The scheme in A′ allows for suggestions that a carbanion intermediate is formed during reaction 6 or that biotin is carboxylated via enzyme-bound CO_2.[15] In mechanism B, deprotonation and carboxylation at 1′-N occur in a concerted manner. In mechanism C, deprotonation occurs subsequent to carboxylation. In other variants of these mechanisms, a tetrahedral adduct of the C-4 carboxyl group of oxaloacetate with the 1′-N of biotin initially forms, and the enolate of pyruvate departs and is subsequently protonated.[5]

Determining the order in which deprotonation and carboxylation of biotin occur requires evaluation of the rates of both processes.

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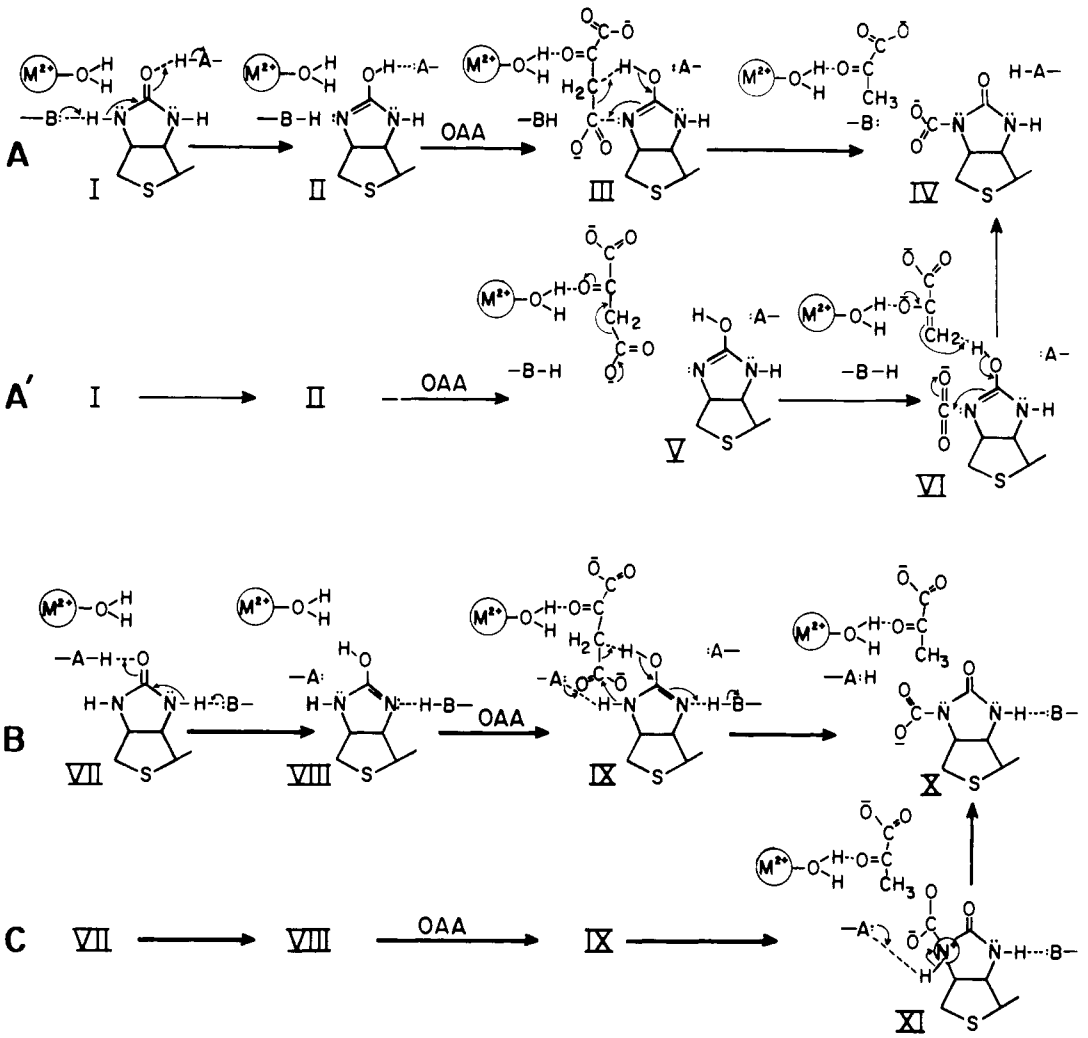

Figure 1. Alternative mechanisms for enzymatic carboxylation of biotin. Deprotonation occurs prior to (A, A'), concerted with (B), or subsequent to (C) carboxylation.

The kinetics of carboxylation have been studied for most biotin enzymes. We now present information on the exchange rates of the amide protons of free biotin, which permits evaluation of mechanisms A, B, and C, and we describe an unusual pH dependence for acid-catalyzed exchange of 1'-NH of biotin and its methyl ester, the mechanism of which is examined by a study of several biotin derivatives. Preliminary reports of this work have been published.[16,17]

Experimental Procedures

Materials. *d*-Biotin was purchased from Sigma, 3'-*N*-methyl *d*-biotin from Hoffmann LaRoche, and desthiobiotin from Nutritional Biochemical Corp., and all other biotin derivatives were synthesized and purified as previously described.[18,19] No contaminants were detected in these compounds by thin-layer chromatography on Whatman MKC18F plates, using 4% water in acetonitrile and 100% acetone as solvents for *d*-biotin and 2% water in acetonitrile and 25% acetone in chloroform as solvents for the other derivatives.

Methods. The compounds were studied in aqueous solutions containing 0.1 M KCl and 10% D_2O, at concentrations of 1 mM for *d*-biotin and 2 mM for all other derivatives.

^1H NMR spectra were obtained on a Bruker WM 250 NMR spectrometer with quadrature phase detection and 16 bit A/D conversion. Suppression of the H_2O peak was achieved through use of the Redfield 2-1-4-1-2 pulse sequence[20] or a 45°-τ-45° hard pulse sequence where τ is a time interval which depends on the chemical shift difference between the resonance of interest and the H_2O resonance.[21]

The exchange rates of the NH protons with those of water were measured by the saturation transfer technique and selective T_1 measurements of the individual NH proton resonances,[22] using pulse sequences and calculations described elsewhere.[23]

Results and Discussion

NMR Spectra of the NH Protons of *d*-Biotin and Derivatives. *d*-Biotin and four derivatives of biotin were utilized in the NH exchange rate studies. The structures of these compounds are given in Figure 2. The downfield region of the ^1H NMR spectrum of *d*-biotin in H_2O contains two resonances (Figure 3), corresponding to the 1'-NH and 3'-NH protons. The 3'-NH resonance was identified on the basis of its absence in the spectrum of 3'-*N*-methyl *d*-biotin (Figure 3). This assignment agrees with the results of homonuclear decoupling experiments.[24] The chemical shift difference between the 1'-NH and 3'-NH resonances may be explained by an orientation of the free carboxylate group toward the ureido ring like that shown in Figure 5. In this

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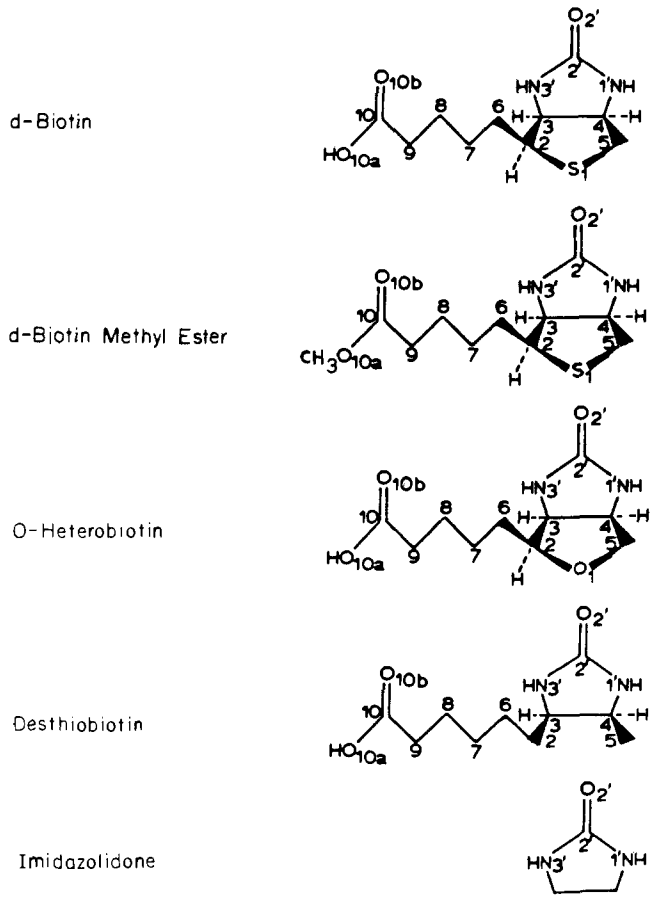

Figure 2. Structures of *d*-biotin and related compounds (adapted from ref 26).

conformation the magnetic anisotropy of the carboxylate group would simultaneously deshield the 3'-NH proton, causing its resonance to shift downfield by 0.054 ppm, and shield the 1'-NH proton, causing its resonance to shift upfield by 0.029 ppm. Analogous effects of carbonyl groups on proton chemical shifts have been observed.[25] The splitting of the 1'-NH resonance of 3'-*N*-methyl *d*-biotin (11 Hz) is apparently due to coupling with its vicinal CH proton; similar coupling (8.6 Hz) is observed with *d*-biotin under conditions in which exchange has been slowed (pH 6, 6 °C). Such couplings are somewhat large, especially at N1, since we calculate from the X-ray coordinates of *d*-biotin[26] dihedral angles of 59° and 41° at the 1'-NH and 3'-NH with their respective vicinal carbon bound protons. Partial decoupling of the NH from the vicinal CH proton resonances was achieved by stepwise irradiation under the intense water resonance. The large couplings may result from smaller dihedral angles in solution as well as from uncertainties in the constants of the Karplus equation for H–N–C–H systems.[27] Resonances of the other biotin derivatives (Figure 3 and 4) were assigned by comparison to *d*-biotin. In the spectra of *d*-biotinyl acetate and *d*-biotin methyl ester (Figure 3), the NH resonances are shifted toward each other, probably due to weaker interactions with the modified side-chain termini.

The chemical shifts of the amide protons of the five compounds were insensitive to pH but did vary with temperature, exhibiting dependencies ranging from −5.4 to −7.6 ppb/°C. These values are comparable to those observed for peptide amide NH protons.[23]

Comparison of Exchange Rates of 1'-NH to Enzymic Carboxylation Rates. The exchange rate of the 1'-NH protons of *d*-biotin and its methyl ester were measured under physiological conditions (Table I). These rates were found to be comparable to k_{cat}, the maximal rates of catalysis by biotin enzymes (Table I), which set lower limits to the rates at which enzyme-bound biotin is carboxylated during catalysis. Therefore, mechanisms in which deprotonation of biotin at 1'-N precedes carboxylation (A or A') appear likely, while mechanisms in which deprotonation is concerted with (B) or follows carboxylation (C) are rendered unnecessary.

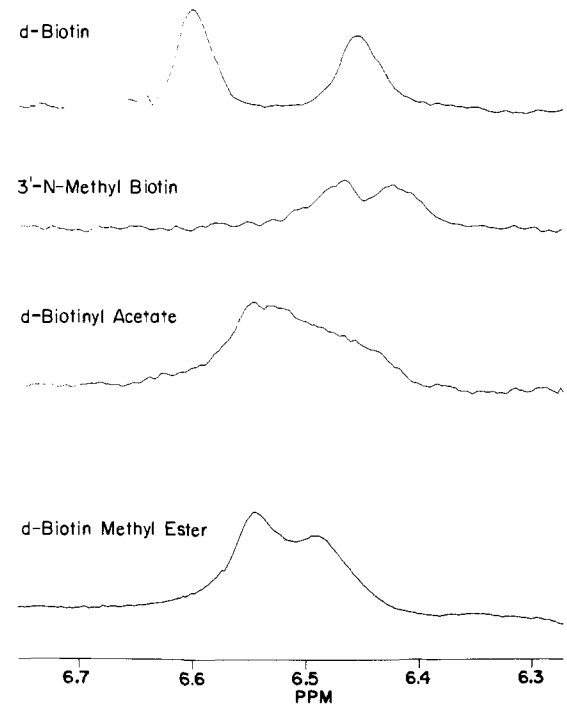

Figure 3. Proton NMR spectra of the NH resonances of *d*-biotin and its derivatives. Solutions contained 1 mM *d*-biotin or 2 mM biotin derivative and 10% D_2O, at pH 6.8 and 10 °C. Spectra were obtained at 250 MHz with a 2–1–4–1–2 pulse sequence with 16-bit A/D conversion, a spectral width of 5000 Hz, quadrature phase detection, an acquisition time of 1.64 s, a relaxation delay of 2.36 s, and 1024 transients (3'-*N*-methyl biotin, *d*-biotinyl acetate) or 4096 transients (*d*-biotin and its methyl ester) of 16 K data points; 1.0 Hz line broadening was applied.

Table I. Comparison of 1'-NH Exchange Rates for *d*-Biotin and Its Methyl Ester to Rates of Enzymatic Carboxylation of Biotin

compound	exchange rate (s^{-1})[a]
d-biotin 1'-NH	58
d-biotin methyl ester 1-'NH	24
enzyme	**k_{cat} (s^{-1})**
transcarboxylase[b]	15
acetyl-CoA carboxylase[c]	40
pyruvate carboxylase[d]	74

[a] Conditions: pH 7.4; 25 °C. [b] From reference 13. [c] From reference 3. [d] From reference 12.

Determination of the Exchange Rates of the Amide Protons of *d*-Biotin and Derivatives at Various pH Values. The exchange rates of the 1'-NH and 3'-NH protons were measured over a range of pH values for the five compounds shown in Figure 2. For both of the NH protons of each compound examined, the dependence of the exchange rate on pH (Figures 6–8) could be described as the summation of base catalysis by OH^- and acid catalysis by H_3^+O as is generally found for amide protons.[28] In Figures 6–8, dashed lines represent the contribution of base and acid catalysis to the observed exchange rates.

Kinetics of Base-Catalyzed NH Exchange. The pseudo-first-order rate constants for base-catalyzed exchange of the 1'-NH and 3'-NH protons of all five compounds exhibited a first-order

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Table II. Acid- and Base-Catalyzed Exchange Rates and pK_A Values for the Amide Protons of *d*-Biotin and Its Derivatives at 25 °C

compound	proton	k_{H^+} (M^{-1} s^{-1})	k'_H (M^{-2} s^{-1})	pK_A[a]	ΔH^* (kcal/mol)	k_{OH^-} (M^{-1} s^{-1})	pK_A[a]
d-biotin	1′-NH	$(4.9 \pm 0.3 \times 10^5)$[b]	$3.8 \pm 1.0 \times 10^{11}$	(−6.0)[b]	5.7	$1.8 \pm 0.1 \times 10^8$	17.4
	3′-NH	$4.9 \pm 0.3 \times 10^5$		−6.0	8.5	$2.9 \pm 0.1 \times 10^7$	18.2
d-biotin methyl ester	1′-NH	$(2.7 \pm 0.2 \times 10^5)$[b]	$7.7 \pm 1.0 \times 10^{10}$	(−6.3)[b]		$7.6 \pm 0.2 \times 10^7$	17.8
	3′-NH	$2.7 \pm 0.2 \times 10^5$		−6.3		$2.9 \pm 0.2 \times 10^7$	18.2
O-heterobiotin	1′-NH	$1.4 \pm 0.2 \times 10^6$		−5.6	8.5	$2.0 \pm 0.2 \times 10^7$	18.4
	3′-NH	$2.0 \pm 0.2 \times 10^5$		−6.7	8.8	$7.4 \pm 0.6 \times 10^6$	18.8
desthiobiotin	1′-NH	$3.1 \pm 1.0 \times 10^7$		−4.2		$6.0 \pm 1.0 \times 10^7$	17.9
	3′-NH	$3.1 \pm 1.0 \times 10^7$		−4.2		$6.0 \pm 1.0 \times 10^7$	17.9
imidazolidone	NH	$1.8 \pm 0.2 \times 10^7$		−4.4		$4.0 \pm 0.2 \times 10^7$	18.1

[a] The pK_A values were calculated with the following equation: $pK_{H^+donor} = pK_{product} + \log (k_{diffusion}/k_{observed})$ where $k_{diffusion} = 10^{10}$ M^{-1} s^{-1}, $pK_{H^+donor} = -1.7$ for H_3O^+, and $pK_{product} = 15.7$ for H_2O. [b] Assumed equal to the value measured directly for 3′-NH.

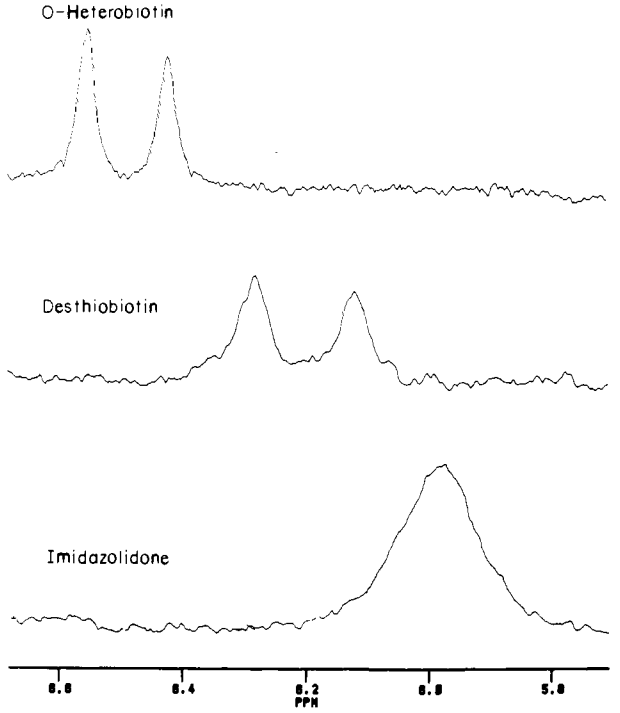

Figure 4. Proton NMR spectra of the NH resonances of the biotin derivatives *O*-heterobiotin, desthiobiotin, and imidazolidone. Solutions contained 2 mM biotin derivative, 0.1 M KCl, and 10% D_2O, at pH 5.9 (*O*-heterobiotin) or pH 6.0 (desthiobiotin, imidazolidone) and 25 °C. Spectra were obtained at 250 MHz with a 2-1-4-1-2 pulse sequence, 16-bit A/D conversion, a spectral width of 5000 Hz, quadrature phase detection, an acquisition time of 1.64 s, a relaxation delay of 2.36 s (*O*-heterobiotin, desthiobiotin) or 0.1 s (imidazolidone), and 128 transients (*O*-heterobiotin, imidazolidone) or 64 transients (desthiobiotin) of 16 K data points; 1.0 Hz line broadening was applied.

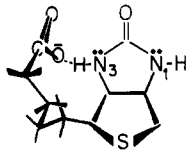

Figure 5. Conformation of *d*-biotin in solution as suggested by the chemical shifts of its NH protons.

dependence on hydroxide ion concentration, as shown by slopes of 1.0 for this relationship in the high-pH region of Figures 6–8. Second-order rate constants (k_{OH^-}) were calculated from these data and are given in Table II.

The values of these rate constants at the catalytically functional 1′-NH were greater than those at 3′-NH for *d*-biotin, *d*-biotin methyl ester,[46] and *O*-heterobiotin. This disparity was not observed on desthiobiotin or imidazolidone. Rate constants at 1′-NH followed the following pattern: *d*-biotin > *d*-biotin methyl ester > desthiobiotin > imidazolidone > *O*-heterobiotin. Among the five compounds, exchange at 3′-NH was faster for desthiobiotin and imidazolidone than for *d*-biotin, its methyl ester, and *O*-heterobiotin. The base-catalyzed amide exchange rates for the five biotin-related compounds were one to three orders of magnitude greater than those reported for urea,[29] *N*-methylurea,[28] *N*,*N*-dimethylurea,[30] and *N*,*N*-methylbenzylurea.[31]

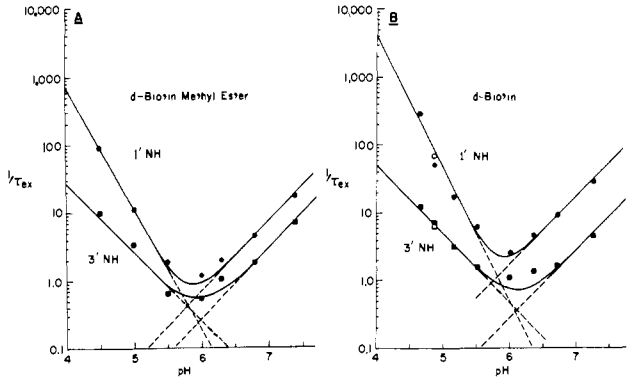

Figure 6. Rates of exchange of amide NH protons with water as a function of pH for *d*-biotin methyl ester (A) and *d*-biotin (B). Exchange rates were measured at 25 °C, using selective T_1 and water saturation transfer techniques as described in the text. Solutions contained 2 mM *d*-biotin methyl ester or 1 mM *d*-biotin, 0.1 M KCl, and 10% D_2O. Open symbols represent measurements obtained for 0.3 mM *d*-biotin. pH was adjusted by the addition of μL amounts of dilute HCl and NaOH. The set of pseudo-first-order exchange rate measurements for each NH proton was fit by a curve representing the sum of two linear functions, corresponding to acid and base catalysis, which are shown as dashed lines. The exchange rates $1/\tau_{ex}$ were fit to the equation $1/\tau_{ex} = k_{OH^-}[OH^-] + k_{H^+}[H^+] + k'_H[H^+]^2$, where k_{OH^-} and k_{H^+} are the second-order rate constants for base- and acid-catalyzed exchange, respectively, and k'_H is a third-order rate constant for acid-catalyzed exchange of 1′-NH. For exchange of 3′-NH, $k'_H = 0$.

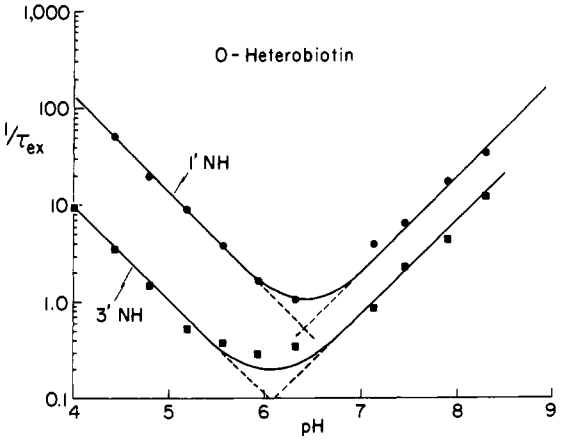

Figure 7. Rates of exchange of amide NH protons with water as a function of pH for *O*-heterobiotin. Exchange rates and rate constants were determined by using the conditions and calculations described for Figure 6. For both 1′-NH and 3′-NH, $k'_H = 0$.

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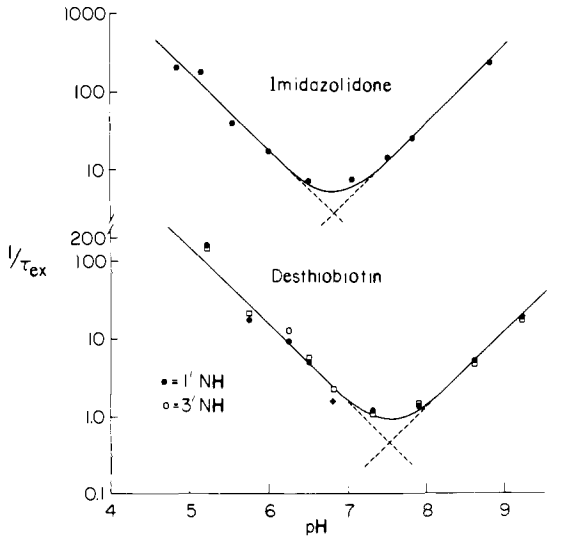

Figure 8. Rates of exchange of amide NH protons with water as a function of pH for imidazolidone (top) at 25 °C and for desthiobiotin (bottom) at 10 °C. All other conditions and calculations were as described for Figure 6. In all cases, $k'_H = 0$.

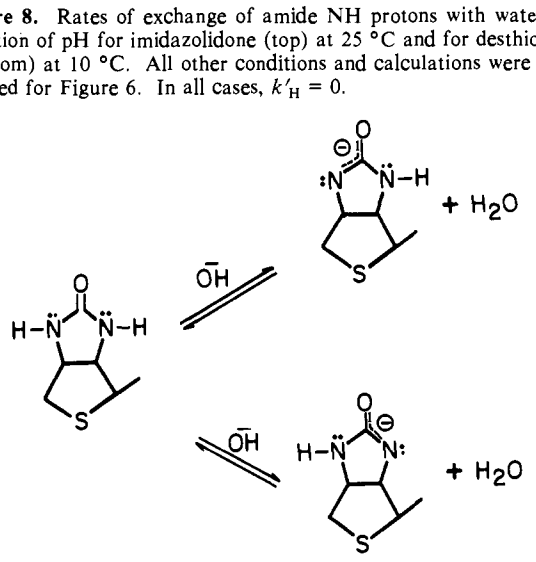

Figure 9. Mechanism of base-catalyzed exchange of the amide NH protons of biotin.

Mechanism of Base-Catalyzed NH Exchange. The first-order dependence of the base-catalyzed exchange rates on OH^- concentration is best explained by a single-step mechanism in which a proton is abstracted at either ureido nitrogen by a hydroxide ion to form water, as shown in Figure 9. This mechanism, which involves dispersion of charge, suggests that base-catalyzed exchange might be enhanced by lowering the ionic strength of the solution. It was observed (data not shown) that for *d*-biotin and its methyl ester, reduction of the KCl concentration from 0.1 M to zero resulted in five- and sevenfold increases, respectively, in the rates of base-catalyzed exchange at 1'-NH and eleven and fourteenfold increases, respectively, in the rates of base-catalyzed exchange at 1'-NH and 11- and 14-fold increases, respectively, at 3'-NH.

The higher rate constant for exchange of 1'-NH over that of 3'-NH, observed for *d*-biotin, its methyl ester, and *O*-heterobiotin, is most likely due to steric interference by the side chain at 3'-N in these compounds which inhibits the approach of OH^- to this position. In the X-ray structure of these compounds, the side chain approaches the 3'-N while in desthiobiotin, which lacks a lower ring, the side chain is not held near the 3'-N.[32] Consequently, in desthiobiotin, and in imidazolidone which has no side chain, a disparity in exchange rates between 1'-NH and 3'-NH is not observed. The absence of this steric effect may also explain why base-catalyzed exchange rate constants at 3'-NH are greater for desthiobiotin and imidazolidone than for the other compounds (Table II).

Variation in the exchange rates at 1'-NH among the five compounds does not correlate with any obvious structural differences. Enhancement of 1'-NH exchange on *d*-biotin by the side-chain carboxyl group is unlikely, since the chemical shift data indicate a similar orientation of this group toward the amide protons of *O*-heterobiotin, yet 1'-NH exchange for this compound is markedly slower than that observed for *d*-biotin. Also, attempts to simulate catalysis by a carboxylate group by substituting 0.1 M potassium acetate for 0.1 M KCl in a sample of *d*-biotin methyl ester did not alter the exchange rate of either NH proton at pH 6.8, even though acetic acid has been shown to enhance the NH exchange of *N,N*-dimethylurea.[30]

Measurements of the exchange rates of the NH protons of *d*-biotin at pH 6.8 at 10, 17, and 25 °C yielded approximate enthalpies of activation ($\Delta H^{\ddagger}$) of 43 ± 8 and 42 ± 3 kcal/mol for the 1'-NH and 3'-NH protons, respectively. These values are substantially greater than the $\Delta H^{\ddagger}$ of 6.8 kcal/mol reported for amide proton exchange in *N,N*-methylbenzylurea,[31] indicating that the faster rate constant for the biotin compounds over acyclic derivatives of urea is due to the lowering of an entropy barrier, presumably due to the rigid ring structure.

Differences in the rate constants may also be interpreted as differences in pK_A values, i.e., with faster base-catalyzed exchange rates resulting from pK_A values closer to the pK_A describing the diffusion-limited protonation of OH^- (15.7).[28,33] The pK_A values of the biotin derivatives obtained from the exchange rates are listed in Table II.

Kinetics of Acid-Catalyzed NH Exchange. The pseudo-first-order rate constants for acid-catalyzed exchange of the 3'-NH protons of all five compounds exhibited a first-order dependence on H^+ concentration, as demonstrated by slopes of 1.0 for this relationship in the low-pH region of Figures 6–8. Second-order rate constants (k_{H^+}) and pK_A values were calculated and are given in Table II. Not surprisingly, the pK_A values for biotin and imidazolidone obtained kinetically in H_2O by extrapolation over 10 p*K* units (Table II) differ somewhat from those measured by titrations in formic acid and $CHCl_3$.[34]

The rate constants for acid-catalyzed exchange of the NH protons of the five compounds show a pattern identical with that observed for base-catalyzed exchange, with the order at 3'-NH being desthiobiotin > imidazolidone > *d*-biotin > *d*-biotin methyl ester > *O*-heterobiotin and that for exchange at 1'-NH being *d*-biotin > *d*-biotin methyl ester > desthiobiotin > imidazolidone > *O*-heterobiotin, although the rate law has changed (see below). As with base-catalyzed exchange, acid-catalyzed exchange rates were consistently lower at 3'-NH than at 1'-NH for *d*-biotin, its methyl ester,[46] and *O*-heterobiotin, with no such disparity observed for desthiobiotin and imidazolidone. Rate constants for acid-catalyzed amide NH exchange for urea and its derivatives[28-31] were greater than those of biotin (3'-NH), its methyl ester (3'-NH), and *O*-heterobiotin but slower than those of desthiobiotin and imidazolidone.

The pH dependence of acid-catalyzed exchange at the 1'-NH was not uniform for the five biotin-related compounds. On *O*-heterobiotin, desthiobiotin, and imidazolidone, exchange of the 1'-NH proton showed a first-order dependence on H^+ concentration. However, for *d*-biotin and *d*-biotin methyl ester, acid-catalyzed exchange of 1'-NH displayed an unprecedented second-order dependence on the square of the H^+ concentration. This is indicated by slopes of 2.0 needed to fit the exchange rate data for 1'-NH on the low-pH side of Figure 6. Lowering the concentration of *d*-biotin from 1.0 to 0.3 mM did not alter this relationship, demonstrating that it was not due to molecular ag-

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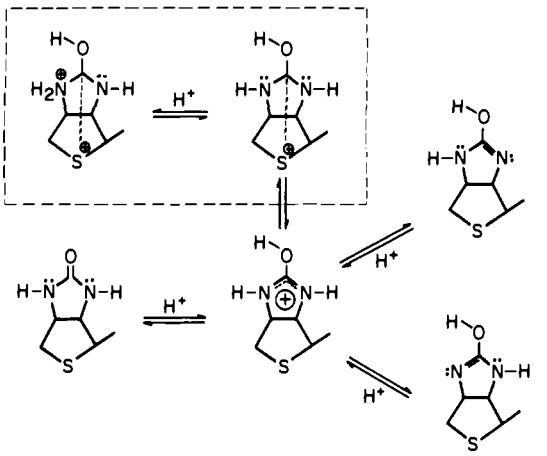

Figure 10. Mechanisms of acid-catalyzed exchange of the amide NH protons of biotin. The pathway enclosed in dashed lines is available only for the sulfur-containing compounds *d*-biotin and its methyl ester and not for *O*-heterobiotin, desthiobiotin, and imidazolidone.

gregation. Hence, an additional third order term $k'_H[H^+]^2[\text{biotin}]$ is required in the rate equations describing the proton exchange at the 1′-NH of *d*-biotin and its methyl ester.

Mechanisms of Acid-Catalyzed Exchange. Acid-catalyzed exchange of an amide proton has been explained by two mechanisms: carbonyl *O*-protonation and amide *N*-protonation.[28,35] Debate exists as to which mechanism is operative for a given type of amide group.[28,35-38] The second-order dependence of acid-catalyzed exchange on $[H^+]^2$ observed for the 1′-NH of *d*-biotin and its methyl ester indicates that two sites of protonation exist on these compounds, the first of which promotes exchange at both 1′-N and 3′-N and the second of which results in exchange only at the 1′-N position. The simplest explanation for the acid-catalyzed amide exchange characteristics of *d*-biotin and its methyl ester is that *O*-protonation catalyzes exchange at both 1′-N and 3′-N and that the *O*-protonated species can undergo additional N-protonation but only at the 1′-N. The overall pathway is shown in Figure 10. N-Protonation at 3′-N is probably prevented due to steric interference from the side chain, which might inhibit the formation of a quaternary nitrogen at this position. This is supported by the slower exchange of the 3′-NH observed for *d*-biotin, its methyl ester, and *O*-heterobiotin, in comparison with desthiobiotin, imidazolidone, and the urea derivatives.

The exchange rate of the 1′-NH of *O*-heterobiotin shows a first-order, not a second-order, dependence on $[H^+]$. This indicates that the presence of sulfur is required to stabilize the doubly protonated species. The simplest explanation for this effect is that following protonation of the carbonyl oxygen, sulfur participates in a transannular interaction with C2′ (Figure 10) preventing delocalization of electron pairs from the nitrogens, thereby allowing additional protonation at the sterically accessible 1′-N position. While we had previously suggested such bonding of sulfur to the carbonyl carbon in enzyme-catalyzed reactions of biotin,[39] the structural evidence pertaining to the ability of biotin to undergo a transannular interaction is inconclusive.[34] X-ray studies of *d*-biotin[26] have shown that, in the crystalline state, S1 and C2′ are 3.68-Å apart, a distance which is only 0.13 Å greater than the sum of the van der Waals radii, 1.70 Å for C2′ and 1.85 Å for S1. By contrast, X-ray studies of *O*-heterobiotin yield a distance of 3.44 Å between O1 and the carbonyl carbon.[40] Although shorter than in biotin, this distance exceeds the van der Waals sum (3.1 Å) by 0.33 Å. In solution, subsequent to protonation of the carbonyl oxygen it is reasonable to suggest that the sulfur could more closely approach the carbonium carbon allowing some bonding to occur. The polar angle between the sulfide plane (C2–S1–C5) and the proposed bond between sulfur and the carbonyl carbon of *d*-biotin is fixed at 32° which is in the range 21 ± 18° observed crystallographically for the interaction of sulfur in thioethers with electrophilic centers.[41]

By measuring the exchange rates of 1′-NH and 3′-NH for *d*-biotin and *O*-heterobiotin at 5, 15, 25, 35, and 40 °C at pH 5.0, the $\Delta H^{\ddagger}$ values for exchange at these positions were calculated for 25 °C and are given in Table II. A comparison of the four $\Delta H^{\ddagger}$ values describing exchange at the 1′-NH and 3′-NH of *d*-biotin and *O*-heterobiotin identifies exchange at the 1′-NH of *d*-biotin as having a singularly low enthalpic barrier. This supports a unique mechanism for acid-catalyzed exchange being operative at the 1′-NH of *d*-biotin, which is the only position among the four at which exchange exhibits a second-order dependence on $[H^+]$.

The fact that protonation of 1′-N can occur following O-protonation only in *d*-biotin and its methyl ester but not in *O*-heterobiotin reflects greater basicity, hence greater nucleophilicity, of 1′-N in the sulfur-containing biotin derivatives.

O-Heterobiotin has generally been found to have no activity or significantly reduced activity in enzymatic carboxylation reactions. Thus, the covalent incorporation of *O*-heterobiotin into the *d*-biotin site of apotranscarboxylase results in an inactive enzyme.[42] The rate of enzymatic carboxylation of free *d*-biotin by the biotin carboxylase subunit of acetyl CoA carboxylase is 5-fold greater than that of *O*-heterobiotin and 14-fold greater than that of desthiobiotin, while imidazolidone is not detectably carboxylated by this enzyme. Hence the role of sulfur in biotin is to increase the basicity and nucleophilicity of the 1′-N, which would promote the carboxylation of biotin. Little or no effect of sulfur on the basicity of the carbonyl oxygen of *d*-biotin is reflected in the pK_A values determined kinetically in H_2O (Table II) or by titrations in $CHCl_3$ or formic acid.[34]

Conclusions

A summary of the present findings which have relevance to the function of biotin enzymes is as follows:

1. Base-catalyzed NH exchange rates on free *d*-biotin are comparable to rates of enzymic carboxylation of biotin, supporting mechanisms in which deprotonation precedes carboxylation (A and A′ in Figure 1).

2. Protonation of the carbonyl oxygen and NH exchange at 1′-N of biotin are facile processes, indicating that the enol form of biotin can be readily attained under enzymatic conditions.

3. The role of sulfur in biotin appears to be to increase the nucleophilicity of the 1′-N facilitating carboxylation at this position. Sulfur may exert its effect by transannular bonding to the carbonyl carbon of biotin.

4. Steric factors reduce the accessibility of the 3′-NH, rendering enzyme mechanisms involving the close approach of a basic group toward this position less favorable (B and C in Figure 1).

5. The diffusion-limited loss of the OH proton of the enol form of biotin, which may reasonably be suggested[33] based on the rapid acid-catalyzed NH exchange (Table II), supports enzymatic mechanisms in which the biotin prosthetic group undergoes minimal migration between substrates in enzymatic carboxylation reactions. The concept of minimal migration of biotin on enzymes was first suggested by NMR studies which determined the proximity of substrates bound to transcarboxylase.[43] On this enzyme, it was found that a migration of ≤7 Å by carboxybiotin would suffice to bring about the transfer reaction. X-ray studies of biotin itself subsequently showed that a comparable 6.9-Å

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translocation of the biotin ring could be accomplished through allowed rotations about two single bonds of the side chain.[40] The minimal migration model received strong support from the observation that transcarboxylase catalyzes a small amount of ^3H transfer between pyruvate and propionyl CoA.[44] If, as seems reasonable on mechanistic grounds, the OH proton of the enol form of biotin is the proton carrier, the diffusion-limited exchange of this proton with solvent[16,33,28] requires a rapid rate of migration of the biotin enol between the two bound substrates, consistent with minimal movement of the biotin ring.

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Cobalt(II) Nitrosyl Cation Radicals of Porphyrins, Chlorins, and Isobacteriochlorins. Models for Nitrite and Sulfite Reductases and Implications for A_{1u} Heme Radicals

Etsuko Fujita, C. K. Chang,[1] and Jack Fajer*

Contribution from the Department of Applied Science, Brookhaven National Laboratory, Upton, New York 11973, and the Department of Chemistry, Michigan State University, East Lansing, Michigan 48824. *Received February 13, 1985*

Abstract: Oxidation of cobalt(II) nitrosyl complexes of porphyrins (P), chlorins (C), and isobacteriochlorins (iBC) yields $Co^{II}NO$ π-cation radicals. The radicals are stable and recyclable to the parent compound without loss of NO. The oxidized species have been characterized by electrochemistry, visible and infrared spectroscopy, and electron spin resonance (ESR). Interest in these intermediates is prompted by the presence of iron Cs or iBCs in enzymes that catalyze the reductions of nitrite to ammonia or nitric oxide (nitrite reductases) and the recent identification of cobalt iBC in sulfite reductases, enzymes which are also capable of nitrite reduction. The optical spectra of the $Co^{II}NO$ radicals resemble those previously obtained on oxidation of $Fe^{II}NOC$ and iBC and lend support to the assignment of those ESR-silent species as $Fe^{II}NOC^+$ and iBC^+ π-cation radicals. As found in other series with the same macrocycles ($Fe^{II}NO$, $Fe^{III}Cl$, Zn, H_2), the $Co^{II}NO$ complexes become progressively easier to oxidize as the porphyrin is saturated (P < C < iBC, easiest). In all three species, the Co–NO bonds remain bent, suggestive of some $Co–NO^-$ character. The C and iBC radicals exhibit ESR features characteristic of "a_{1u}" radicals that include spin delocalization onto the metal. Similar spin profiles may rationalize the ESR spectra of presumed "a_{1u}" porphyrin radicals in oxidized chloroperoxidase, catalase, and cytochrome P450.

Prosthetic groups comprised of iron isobacteriochlorins (sirohemes) and chlorins (hemes d) have been identified in several enzymes that catalyze the multielectron reduction of nitrite to ammonia[2] (assimilatory nitrite reductases, sirohemes) or its one-electron reduction to nitric oxide[3] (dissimilatory reductases, hemes d). The possibility that $Fe^{II}NO$ complexes occur in the catalytic cycles of the enzymes[4] led us[5] to a comparative study of the redox chemistry of $Fe^{II}NO$ complexes of porphyrins (P), chlorins (C), and isobacteriochlorins (iBC). No distinctive features that favored the biological selection of one macrocycle over the others were found either in their reduction potentials or in the binding of NO. However, the progressive saturation of the porphyrin framework made their $Fe^{II}NO$ complexes easier to oxidize, resulting in a shift from metal-centered oxidation in the porphyrin to macrocycle oxidations in the chlorin and isobacteriochlorin. The NO substrate became less labile in the oxidized C than in P, and still less in iBC. The Fe–NO bond remained bent in the oxidized C and iBC but was linear in the P. Because linear and bent M–NO bonds may be formally considered[6] as $M–NO^+$ and $M–NO^-$, respectively, a bent Fe–NO bond may therefore be more susceptible to protolytic reactions that eventually generate ammonia in the sirohemes. If electron transport in the enzymic cycle occurs via oxidized macrocycle transients, as previously suggested,[7,8] then the biological choice of macrocycle would thus allow control of oxidation site (metal vs. macrocycle) and of the reactivity and residence time of the NO substrate at the catalytic site.

Our conclusions[5,9] regarding the $Fe^{III}NOP^+$ species, reached on spectroscopic evidence, were confirmed by Scheidt et al.,[10] who

* Address correspondence to this author at Brookhaven National Laboratory.

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