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Role of CO_2 in Proton Activation by Histidine Decarboxylase (Pyruvoyl)†,‡

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ABSTRACT: The amino acid decarboxylases that use an intrinsic pyruvoyl cofactor have been viewed in terms of the pyridoxal-P paradigm whereby a Schiff base is formed between the enzyme-bound cofactor and the substrate, setting up a cation sink for electrons of the C_α–CO_2^- bond, ejecting CO_2, and the reversal of these steps with a proton with overall *retention* stereochemistry. With histidine decarboxylase (pyruvoyl) it is found that the presence of CO_2 is required for T-exchange between histamine and water. Since the forward reaction including formation of the C–H bond does not require added CO_2, it might be assumed that the CO_2 that is formed in the fragmentation step is retained by the enzyme perhaps to assist in proton transfer. No such requirement for CO_2 has been reported for the pyridoxal-P-dependent decarboxylases which are generally thought to liberate CO_2 prior to proton transfer. In seeking a connection between bound CO_2 and proton transfer in the histidine decarboxylase reaction, one is reminded of the carboxybiotin enzymes also known for an invariant stereochemistry of retention and for the requirement that the biotin be in the carboxylated form for H-exchange to occur. Perhaps the bound CO_2 of histidine decarboxylase forms a carbamate by addition to Lys155 or to an amide group of the active site. The new carboxy group could then be the vehicle for protonating the carbon from which it originated, giving overall retention of the stereochemistry at the α-C. Carboxybiotin may serve a similar role in reverse, using the carboxylate to abstract the proton from the substrate, giving the carbanionic center to which carboxybiotin-derived CO_2 would then be transferred. Two other models are considered to explain the requirement for CO_2 for proton exchange from histamine by the decarboxylase. One of these assumes a conformational effect of CO_2 that leads to proper orientation of the proton donor. This is implied by a significant effect of CO_2 on the apparent affinity of imidazole comparing its K_i in the decarboxylase and the T-exchange reactions under the same conditions. In another model CO_2 is required for the complete reversal of reaction, histamine + CO_2 → histidine. This model requires that the abstracted T remains sequestered on the enzyme during formation of all normal reaction intermediates and can only be liberated at some step subsequent to carboxylation. This will require an extraordinary immobilization of the abstracted proton in the absence of CO_2, $k_{exchange}$ $<10^{-5}$ s^{-1}.

Histidine decarboxylase of *Lactobacillus* 30a is the best studied amino acid decarboxylase for which an intrinsic pyruvoyl residue serves as a cofactor, filling the role of pyridoxal-P in other enzymes of this class [for reviews see Boeker and Snell (1972), Gallagher et al. (1989), Recsei and Snell (1984), and Van Poelje and Snell (1990)]. Notable are extensive steady-state and inhibition studies (Recsei & Snell, 1970; Alston & Abeles, 1987), heavy atom kinetic isotope effect studies (Abell & O'Leary, 1988), specific amino acid replacements (Gelfman et al., 1991), a 2.5-Å resolved X-ray map of the enzyme alone and with histidine methyl ester, and a 3-Å map with histamine (Gallagher et al., 1989). The substrate binding site is composed of an imidazole binding pocket contributing both hydrophobic and H-bonding interactions and a substrate carboxyl binding pocket made up of seven hydrophobic residues and Lys155 and Glu197 as the only conspicuous acid/base reagents that might contribute to enzyme-mediated proton transfer to the α-carbon.

The mechanism shown in Scheme I [adopted from Recsei and Snell (1984)] uses the carbonyl of the N-terminal pyruvoyl residue to form a Schiff base with histidine, SB1.[1] Decarboxylation of SB1 produces CO_2 and the central intermediate, X, the enzyme-bound imine of histamine. The Schiff base of histamine, SB2, is formed by protonation, and histamine is liberated by its hydrolysis. Both SB1 and SB2 were established by chemical identification of sodium borohydride trapped products derived from a single incubation with histidine (Recsei & Snell, 1970). Observation of the derivative of the histidine Schiff base indicates that decarboxylation is a slow step. Consistent with this is a substantial V/K ^{13}C isotope effect

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‡ We dedicate this paper to Harland G. Wood (1907–1991) in appreciation of his many elegant contributions to our understanding of the action of CO_2 in metabolism together with a distinguished career of teaching and leadership.

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[1] Abbreviations: SB1 and SB2, Schiff base intermediates of Scheme I; HEPES, *N*-(2-hydroxyethyl)piperazine-*N*′-2-ethanesulfonic acid.

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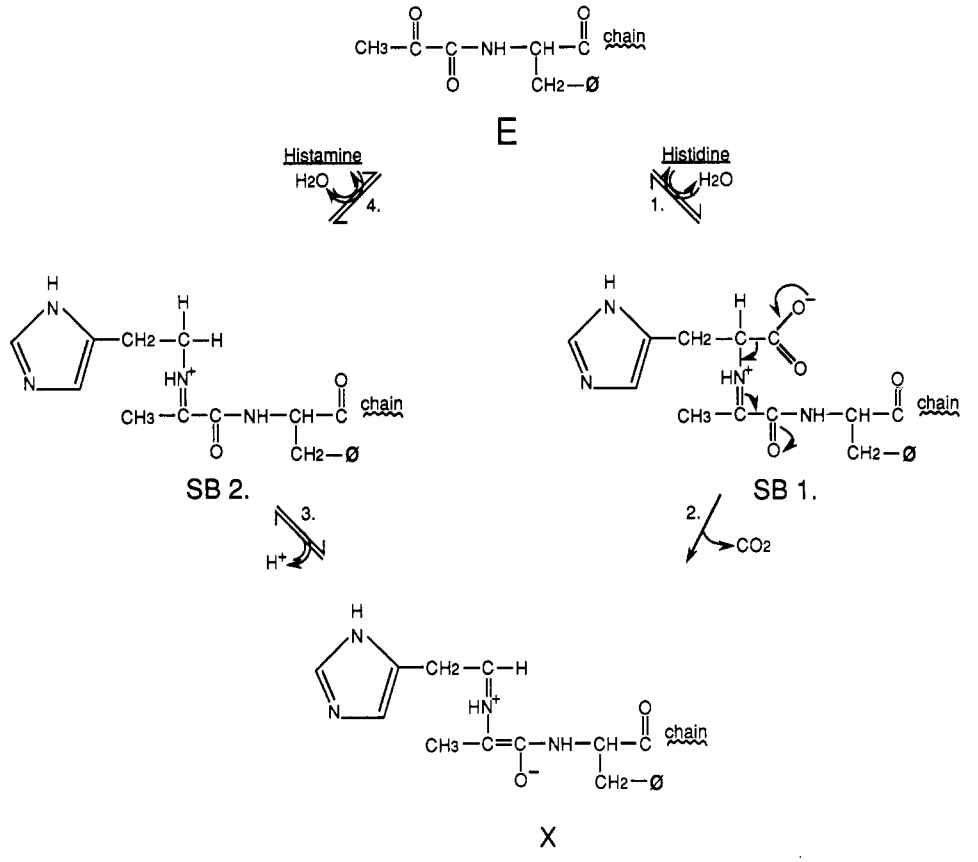
Scheme I



(Abell & O'Leary, 1988) which requires that the steps preceding decarboxylation be more rapid than the fragmentation step. The immediate fate of the "first product", CO_2, is unknown. From the crystallographic analysis of histidine decarboxylase with histidine methyl ester, the histidine carboxyl group is buried among hydrophobic residues, providing a possible binding site for CO_2. Since the proton approaches from the same face of the imine C of the intermediate (Chang & Snell, 1968; Battersby et al., 1980; Santaniello et al., 1981), Scheme I shows CO_2 leaving the enzyme before protonation can occur. That this may not be the case will be argued from the observations that CO_2 is required for exchange of T from (T)histamine.

In considering the possible role of CO_2 in the T-exchange reaction, we note that (T)histamine produced from histidine had the same specific radioactivity (±10%) as the medium. This was observed over the pH range 4.5–8.0. Failure to discriminate between T and H in a reaction in which a medium-derived proton is transferred to C can result if the proton-transfer step is not at all rate determining relative to free product formation. Alternatively, if the proton to be transferred cannot exchange with the medium at a rate sufficient to compete with transfer, it will be committed to product formation so that after a lag of only a few turnovers T and H will appear in product at the solvent ratio. This slow exchange may be due to a high apparent pK_a of the donor group (a sticky proton) or to inaccessibility of the abstracted proton to protons with which to exchange (a blocked proton).

Experimental Procedures

Materials

Histidine decarboxylase from *Lactobacillus* 30a was kindly provided, on two occasions, by Professor E. E. Snell, making these studies possible. Before each experiment a sample of enzyme in buffer (10 mM ammonium acetate, pH 4.8, 1 mM EDTA) was centrifuged through a spun-dry G-25 column that had been equilibrated with the same buffer. At 24 °C the enzyme had an activity of 48 s^{-1} following T uptake from water or $^{14}CO_2$ formation from (1-^{14}C)histidine in ammonium acetate buffer (pH 4.6, 0.2 M), using a 280-nm extinction coefficient of 16.1 for a 1% solution of protein (Riley & Snell, 1968).

(1-T)Histamine (2.3×10^7 cpm/μmol) was produced by complete decarboxylation of histidine in (T)water at pH 4.8. It was isolated from Dowex 50-H^+, using 0.5 N triethylamine. The specific activity was very close to that of the water. Similar experiments at pH 7.5 and 8.0 (0.2 M HEPES) failed to show any isotopic discrimination in forming labeled product.

Methods

Detritiation of (T)histamine was followed by passing a TCA-stopped reaction through a small column of Dowex 50-H^+, counting the effluent and water wash of the column. The reaction rate was determined using $v = t^{-1}$(histamine) ln $(1 - f)$, where f is the fraction of counts exchanged.

Addition of CO_2 to incubations was made as follows: The incubations, generally in 10 μL in 12- × 75-mm glass test tubes, were sealed in 290-mL centrifuge bottles with a known weight of solid CO_2, using an O-ring to withstand the pressure developed. The concentration of CO_2 was determined from the weight of added CO_2, the volume of the system, and the solubility constant of 33 mM/atm at 25 °C (Umbreit et al., 1959). Bottles were weighed periodically during the prolonged incubation as assurance against leakage.

To determine the T/H isotope effect in the exchange reaction, (T)histamine (5 mM) was incubated with enzyme, CO_2, and phosphate buffer in D_2O, pD = 7.5. Appearance of T in water was compared with the loss of H from the C-1 position of histamine that was reisolated on Dowex 50-H^+ as above and analyzed for ^{1}H by NMR on a Bruker AM-300 at

Table I: CO_2 Activates (T)Histamine/Water Exchange[a]

additions (mM)	T into water (cpm)
minus enzyme	165
+CO_2 (180)	3260[b]
+CO_2 (180) + HCO_3^- (50)	2840
+CO_2 (180) + imidazole (5)	225
no CO_2 (N_2)	201

[a] Each 10-μL incubation contained (T)histamine (378 μM, 23 800 cpm/nmol) in HEPES (0.2 M, pH 7.5) and 0.15 nmol of enzyme when present. Reactions were terminated at 125 min. [b] The CO_2-activated rate is 1.2×10^{-4} s^{-1}.

300 MHz using the imidazole protons as an internal standard. We are grateful to Dr. Steven Seeholzer for the NMR analyses.

Kinetic studies and enzyme assays in the forward direction were done by following the loss of ^{14}C from incubations of (1-^{14}C)histidine. The acid-quenched samples were counted directly after flushing briefly with N_2. This method, even with only 10–20% of substrate turnover, gave linear rates and good reproducibility, making it more convenient than the conventional assay based on the collection of $^{14}CO_2$.

Results

Requirements for T-Exchange from (R)-(1-T)-Histamine. As discussed above, the absence of discrimination between T and H in the formation of histamine in (T)water may result from rapid proton equilibration between the medium and the enzyme–histamine Schiff base, or from very slow equilibration from the carbanion intermediate, E_X^T. It has been established that the decarboxylation and protonation steps are not concerted but sequential since the large ^{13}C discrimination in forming CO_2 was not changed in D_2O (Abell & O'Leary, 1988). To investigate the possibility of rapid proton equilibration between water and bound histamine (rate-limiting release of histamine), the rate of that exchange was measured over the pH range 4.5–7.5. No formation of (T)water from (T)histamine was observed. In an attempt to reconstruct the full ternary complex, the experiment was repeated with added CO_2. In this way the absolute requirement for CO_2 was found (Table I).

The CO_2-dependent exchange rate was shown to be constant for at least 18 h at pH 7.5 in 0.2 M HEPES. Normal hyperbolic kinetics were observed in the T-exchange reaction at pH 7.5 both for CO_2 ($K_m \simeq 62$ mM) at 0.47 mM (T)histamine and for histamine ($K_m \simeq 0.4$ mM) at 30 mM CO_2 (Figure 1, panels A and B). The latter, which represents an equilibrium value, is ~25% tighter than the K_i value reported for histamine in the decarboxylation assay at pH 4.8 (Recsei & Snell, 1970). The T-exchange rate predicted for infinite concentration of both substrates is about 10^{-3} s^{-1}. No isotope discrimination between (T)- and (H)histamine was observed in the exchange reaction in D_2O when followed by radioactivity appearing in water and the loss of H determined by H-NMR analysis of recovered histamine.

CO_2-dependent detritiation of histamine could be shown over the whole pH range studied, pH 4.6–8.0. Bicarbonate (40 mM) at pH 7.5 in an open tube did not replace CO_2 significantly.

Imidazole, a weak competitive inhibitor of the forward reaction at pH 4.8, K_i = 3.2 mM (Chang & Snell, 1968), was more inhibitory than expected when measured as an inhibitor of T-exchange at pH 7.5 (Table I). This impression was borne out by a comparison of K_i values made at pH 7.5. Imidazole was competitive for both histidine decarboxylation, K_i ~0.70 mM, and histamine detritiation, K_i ~0.03 mM (Figure 2, panels A and B), a 23-fold increase in affinity in the presence of CO_2.

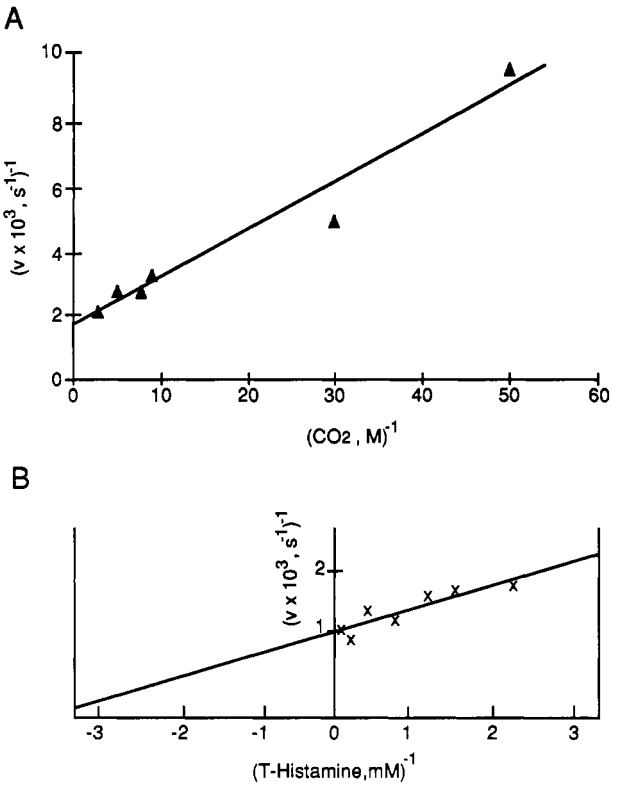


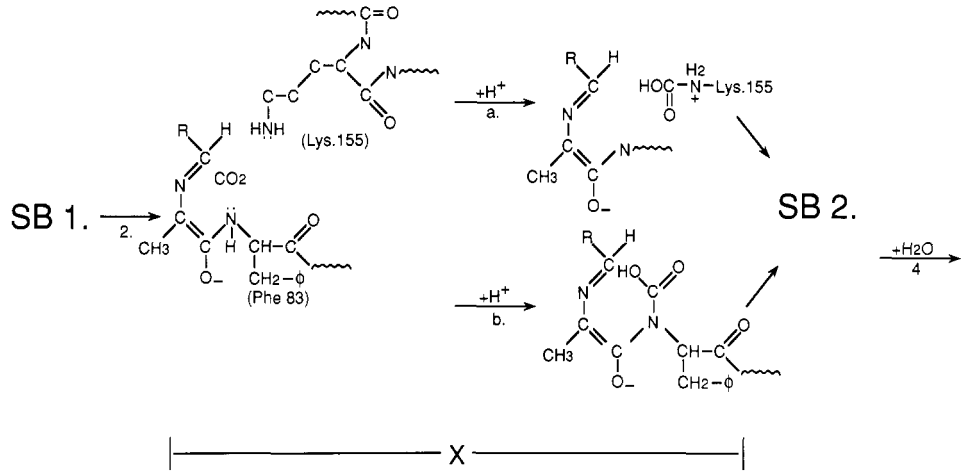
FIGURE 1: CO_2 and histamine concentration dependence for (T)-histamine/water exchange: (A) CO_2 was varied from 19 to 400 mM with (T)histamine (0.47 mM), enzyme (7.4 μM), and HEPES (0.25 M, pH 7.5). After 15 h, the 10-μL incubation was acidified and (T)water determined. $K_m(CO_2)$ = 62 mM ± 25%, $k_{cat} = 5 \times 10^{-4}$ s^{-1} ± 10%. (B) (T)Histamine was varied from 0.39 to 35 mM with CO_2 (30 mM), enzyme (12 μM), and HEPES (0.05 M, pH 7.5). T in water was determined after 120 min. K_m(histamine) = 0.42 ± 0.15 mM, $k_{cat} = 10^{-4}$ s^{-1} ± 10%.

The forward rate, expected to be cooperative with respect to histidine above pH 7.0 (Recsei & Snell, 1970), was found instead to have simple hyperbolic concentration dependence using HEPES at pH 7.5 (Figure 2A). An observation of possible significance is a 3–4-fold increase in exchange rate with increasing HEPES from 20 to 200 mM. This may be ascribable to improved pH control at the higher buffer level except that normal hyperbolic kinetics over a wide CO_2 concentration range (Figure 1A) makes this seem unlikely considering the sensitivity of the K_m of histamine to pH indicated by K_i at pH 4.8 of 11 mM (Recsei & Snell, 1970) and K_m at pH 7.5 of 0.42 mM at 30 mM CO_2 in the detritiation assay (Figure 1B). Ionic strength was controlled with NaCl in these studies, a possibly unfortunate choice, since the exchange rate decreased ~30% in going from 23 to 230 mM NaCl.

Discussion

There is great interest in establishing the mechanisms of interaction of CO_2 with enzymes. No evidence for a binary E·CO_2 noncovalent complex could be found with carbonic anhydrase (Bertini et al., 1987) or ribulose-1,5-P_2 carboxylase (Pierce et al., 1986) with affinities tighter than 1 M and 5 M, respectively. It was with this in mind that we approached the problem of how CO_2 facilitates a proton abstraction from (T)histamine catalyzed by histidine decarboxylase. Unlike the well-known use of CO_2 to activate ribulose-1,5-P_2 carboxylase, which depends on formation of a carbamate (Lorimer & Miziorko, 1980), there is no evidence for an activation role for CO_2 in the decarboxylase reaction per se. It therefore

Scheme II



seemed likely that the CO_2 requirement reflected a role of the CO_2 produced by the decarboxylase action on the proton-transfer step required to produce histamine.

The possibility that the CO_2 effect on exchange could be due to a chemical process that is irrelevant to the enzyme mechanism seems remote for a number of reasons. An irrelevant chemical process would be expected to be rate limiting in the proton abstraction step, yet no T/H isotope discrimination was observed. Using (T/H)histamine in D_2O, both CO_2 and histamine show saturation kinetics consistent with a termolecular complex. CO_2 binding to the enzyme alters the active site, increasing the affinity for imidazole. Finally, the exchange reaction observed is specific for the same proton of histamine that is derived from solvent in the decarboxylation of histidine (Battersby et al., 1980). The requirement for CO_2 argues that the exchange activity is not caused by a contaminating enzyme.

The stereochemistries of two other pyruvoyl type decarboxylases are known also to go with retention: adenosylmethionine decarboxylase (Allen & Klinman, 1981) and phosphatidylserine decarboxylase (No et al., 1988). There can be no metabolic pressure to favor retention in the evolution of enzymes of this class given the achirality of the amine carbon of the products. This can also be said for the pyridoxal-P-dependent decarboxylases and the biotin-dependent transcarboxylations. The conventional, minimal-motion, view of amino acid decarboxylases would have CO_2 leaving the enzyme before proton transfer to the carbanion intermediate (Scheme I). If these steps are independent, why should stereochemistry be conserved? The finding that CO_2 is required for T-exchange by histidine decarboxylase raises the possibility that some form of chemical coupling is responsible for the stereochemistry of this enzyme and suggests that similar experiments may be informative with the other decarboxylases.

Failure to observe discrimination between (T)- and (H)-histamine in the exchange reaction indicates that dissociation of the donor, not the proton abstraction step, is rate limiting. Three proposals will be considered to explain the CO_2 requirement. They may be designated as follows: the conformational effect model, the carbamate model, and the blocked proton model. The requirement for CO_2 could be consistent with the reaction stereochemistry if in the forward direction the CO_2 generated causes a conformational change necessary for the proper orientation of the proton donor. In the reverse reaction with (T)histamine this induced effect is required to orient the proton acceptor. An ability of CO_2 to induce an effect on the remote binding site of imidazole (implied from K_i values, Figure 2) would be consistent with this possibility.

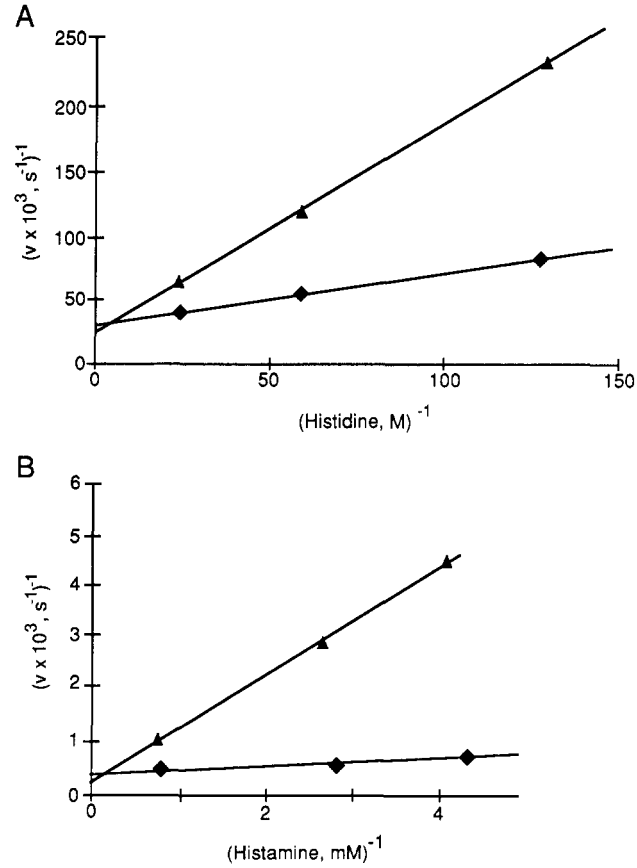
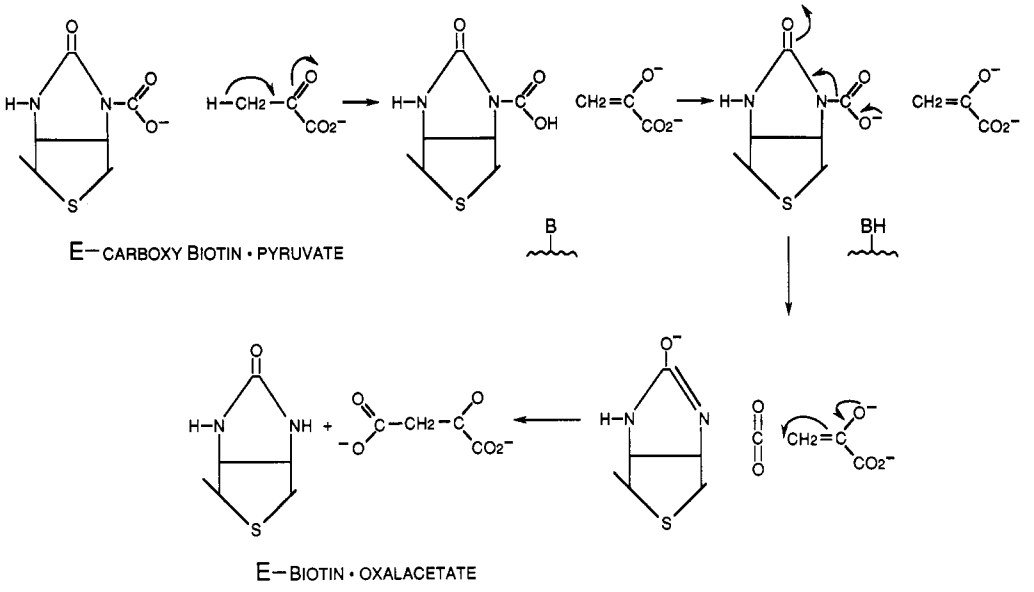


FIGURE 2: Imidazole as an inhibitor of decarboxylation (A) and detritiation (B) reactions: (A) Imidazole (0 or 2.5 mM, ◆ or ▲) in 50 μL containing (1-^{14}C)histidine (4–40 mM, 5 × 10^3 cpm), enzyme (2 pmol), and HEPES (50 mM) was incubated at 22 °C for 10 min. The loss of ^{14}C from the reaction mixture quenched with acid was used to calculate K_m(histidine) = 9.63 ± 0.72 mM, K_i(imidazole) = 0.72 ± 0.06 mM, and k_{cat} = 45 ± 1 s^{-1}. (B) Imidazole (0 or 0.33 mM, ◆ or ▲) in 15 μL containing (T)histamine (0.09–1.3 mM, 2 × 10^4 cpm), CO_2 (127 mM), enzyme (118 pmol), and HEPES (130 mM, pH 7.5) was incubated at 22 °C for 97 min. T counts exchanged into water were used to calculate K_m(histamine) = 0.16 ± 0.04 mM, K_i(imidazole) = 0.029 ± 0.008 mM, and k_{cat} = 2.77 ± 0.23 × 10^{-4} s^{-1}. [Data for 90 μM (T)histamine fell on the lines drawn but are not shown for ease in presentation.] Standard deviations were determined by a nonlinear regression program.

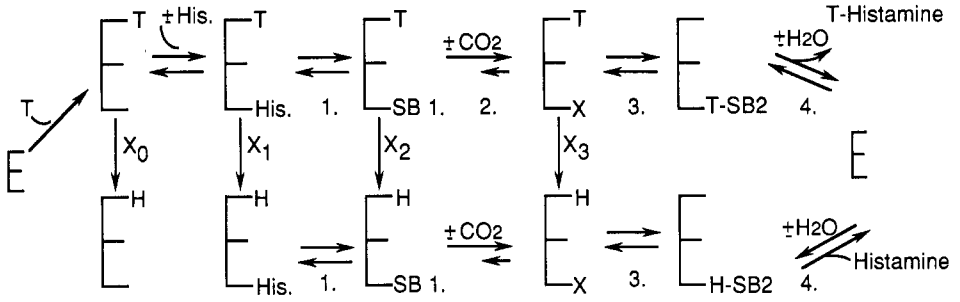
Given the poor binding properties of CO_2 noted above, it seems unlikely that the CO_2 effect would come from simple association.

Instead of the conformational coupling model, it may be proposed that CO_2 that is formed in the decarboxylation of

Scheme III



Scheme IV



histidine plays a direct mechanistic role in the protonation step. Suppose the hydrophobic environment at the *re* face of the imine intermediate prevents CO_2 escape and promotes formation of a carbamate adduct with nearby Lys155-NH_2 or with the amide N derived from Phe-83 (Scheme IIa,b). The carbamate anion would then transfer a proton from a site on the enzyme as required by T-capture results (Rose and Kuo, unpublished results) to the intermediate with the required stereochemistry.

Carboxylation of an amine residue by the Schiff base of an amino acid, SB1, may be no less efficient than the carboxylation of biotin by a β-keto acid on an enzyme such as pyruvate carboxylase. All biotin-dependent carboxylation reactions go with a stereochemistry of retention. Furthermore, with all enzymes that have been studied the biotin must be carboxylated for proton abstraction to occur (Prescott & Rabinowitz, 1968; Mildvan et al., 1966; Kuo & Rose, 1982). A concerted mechanism was initially proposed (Rêtey & Lynen, 1965) but has been ruled out by evidence demonstrating a carbanion intermediate in propionyl CoA carboxylase (Stubbe et al., 1980) and in transcarboxylase (Kuo & Rose, 1982). To explain why all biotin enzymes require the biotin to be in the carboxylated form to achieve proton abstraction from the substrate in what is purported to be a stepwise mechanism, it may be proposed (Scheme III) that the basic residue responsible for the proton abstraction is the carboxylate group of the carboxybiotin itself, solution pK_a = 4.25 (Tipton & Cleland, 1988). The abstracted proton is next transferred to the enzyme, and the carboxylate group, released as CO_2, does not reach the medium but is captured (Sauers et al., 1975) by the carbanionic C of the intermediate. Suggestion of a basic group, B, presumably a residue of the biotin-carrying subunit, is necessary to explain the observation made with transcarboxylase (Rose et al., 1976) of T transfer between pyruvate and propionyl CoA in substitution for the carboxyl of methylmalonyl-CoA.

The "blocked proton model" can be discussed in reference to Scheme IV. In this explanation T-exchange from (T)-histamine requires CO_2 because the abstracted T cannot escape from E_X^T except by reverse of the decarboxylation step, i.e., $kx_3 \ll k_{-2}$. This model provides a reasonable explanation for the low maximum rate of T-exchange, $\sim 10^{-5} k_{cat}$, since by depending on k_{-2} the exchange process would involve all of the steps required to reverse the exergonic decarboxylation. The hydrolysis of SB1 and dissociation of E·His are rapid so that T may dissociate from the free enzyme.

At this point the data are insufficient to choose among the models mentioned or to rule out any of them. The maximum T-exchange rate with CO_2 present, $\sim 10^{-3}$ s^{-1}, is certainly too slow for positional exchange with H of a polyprotic base such as Lys155 to be the basis for T-exchange. Also it provides no role for CO_2 in the exchange. In the absence of CO_2 the exchange rate is <1% of 10^{-3} s^{-1} (Table I). In the blocked proton model in which CO_2 is not required for T abstraction from the (T)histamine, but only for its subsequent transfer to the medium, the stability of E_X^T toward exchange would have to be considerable. Given k_{cat} for exchange of $<10^{-5}$ s^{-1} in the absence of CO_2, if E_X^T were only 10^{-4} of the total enzyme in the exchange equilibrium, its half-life toward exchange would have to be of the order of 10 s. This seems like an extraordinary immobilization to occur in a complex that is presumed to be accessible to CO_2 of the medium.

An important role for Glu197 is suggested by the 8000-fold lower k_{cat} of the Glu197Gln mutant (McElroy & Robertus, 1989). Glu197 with pK_a elevated by a hydrophobic environment is best oriented to act as the proton donor according to the structural studies with histidine methyl ester (Gallagher, 1989). In the ionized form Glu197 might assist the decarboxylation by charge repulsion (Gallagher, 1989; McElroy & Robertus, 1989). As with the carbamate model, Glu197 could act as a base to ionize the substrate carboxyl. Return of this proton to the carbanion intermediate after decarboxylation would give retention stereochemistry. However, isotope trapping experiments with enzyme in (T)water chased by histidine in normal water (Rose and Kuo, unpublished results) indicate that the proton that forms the histamine is derived from the enzyme before mixing with histidine.

A critical test of the role of CO_2 in causing the exchange, whether as a carbamate resulting in a conformational change, as a carbamate base abstracting the proton, or as CO_2 completing the carboxylation reaction, would be possible with an enzyme which, in the decarboxylation direction, showed discrimination against T of water. In this case exchange of T at x_3 (Scheme IV) could not be so completely blocked as in the histidine decarboxylase reaction. A CO_2 requirement for exchange in this case could not be explained as a need to complete the overall reaction. Tests are in progress with adenosylmethionine decarboxylase (pyruvoyl), which shows a 4.5-fold discrimination in the formation of product in (T)-water and which like histidine decarboxylase (pyruvoyl) was reported not to catalyze proton exchange from the decarboxylated product (Allen & Klinman, 1981).

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