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Mechanisms for the Enzyme-Catalyzed ATP-Dependent Carboxylation of Biotin Involving Phosphorylated Tetrahedral Intermediates

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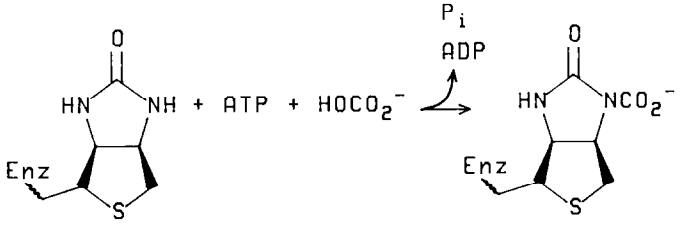
The mechanism of enzyme-catalyzed carboxylation of biotin is considered in terms of the order of bond-forming reactions among the three enzyme-bound substrates: ATP, biotin, and bicarbonate (producing $N_{1'}$-carboxybiotin, ADP, and inorganic phosphate). A route is proposed in which the addition of biotin to bicarbonate generates an adduct which reacts with ATP in either one or two steps. This adduct decomposes to give $N_{1'}$-carboxybiotin and inorganic phosphate. The mechanism is consistent with data from studies of various enzymatic reactions and reaction patterns revealed by studies of intramolecular model reactions.

Biotin-dependent carboxylases catalyze the exchange of an equivalent of carbon dioxide from dissolved bicarbonate for the proton attached to $N_{1'}$ of biotin. In the same reaction, ATP is cleaved to ADP and inorganic phosphate (*1*, *2*). The



reaction poses a significant mechanistic question which is related to other processes where ATP cleavage "drives" another reaction: How does the net cleavage of ATP lead at the same time to the formation of a carbon–carbon bond? Although a number of mechanisms have been proposed for the carboxylation of biotin, none accommodates all the experimental data without ad hoc assumptions (*1–3*). However, we find that a systematic evaluation reveals an additional set of possible mechanisms which are consistent with reported data and which overcome some of the problems with other mechanisms. In order to present the proposals in context, we review some key observations.

1. Labeling studies with ^{18}O bicarbonate showed that during the course of the biotin carboxylation reaction, an oxygen atom from bicarbonate is transferred into the inorganic phosphate product (*4*).

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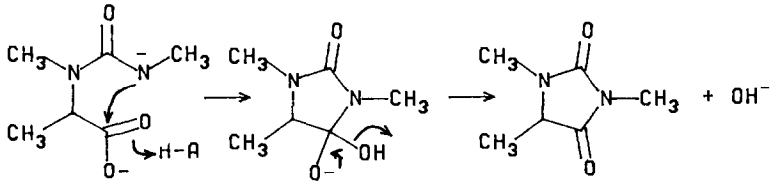
2. Attempts to observe isotope exchange reactions in a less than fully constituted system have shown that partial reactions do not occur. In particular, there is no ADP–ATP exchange (*3, 5*).

3. Experiments designed to detect biotin carboxylase-catalyzed positional isotope exchange of the terminal phosphate of ATP in the absence of biotin (which would indicate a separate reaction between ATP and bicarbonate) gave negative results (*6, 7*). The most straightforward interpretation of the lack of exchange is that all reactants are involved in a common transition state on the enzyme. Alternatively, it can be argued that product release is relatively slow, there is product inhibition or the phosphate groups are tightly bound, preventing positional isotopic exchange (*3, 6*).

4. The net stereochemistry of the displacement of the terminal phosphate of ATP to produce inorganic phosphate occurs with inversion during the reaction catalyzed by pyruvate carboxylase (*8*).

5. Biotin-dependent enzymes catalyze a number of mechanistically informative alternative reactions. Biotin carboxylase catalyzes the formation of ATP from ADP and carbamyl phosphate in the absence of biotin (*9*). This supports the idea that the enzyme promotes transfer of phosphate from an intermediate (for which carbamyl phosphate can serve as a surrogate) to ATP (*10*). In the absence of biotin, the enzyme also catalyzes the bicarbonate-dependent hydrolysis of ATP with concomitant transfer of oxygen from bicarbonate to the inorganic phosphate produced (*6, 7*).

6. Studies of the reactions of molecules containing the functional groups involved in the carboxylation of biotin have identified some reactivity patterns but do not permit one to decide which provides a valid analogy (*2, 3*). The compound expected to be formed from the displacement of ADP from ATP by bicarbonate (carboxyphosphate) will have a short lifetime ($t_{1/2}$ = 70 ms) and thus could provide a local concentration of unsolvated carbon dioxide to react with $N_{1'}$ of biotin (*11*). Recent studies of proton exchange reactions at $N_{1'}$ have shown that the conjugate base of the urea group of biotin can be a viable intermediate (*12, 13*). This could be generated in a transition state in which the proton is removed from nitrogen in concert with attack of the nitrogen center upon the source of the incipient carboxyl carbon of *N*-carboxybiotin (*9*). Studies of the hydrolysis of phosphonate esters adjacent to ureas indicate that it is reasonable to expect biotin to function as a nucleophile toward ATP (*14, 15*). The species thus generated would be expected to react with bicarbonate in analogy to mechanisms proposed for the action of DCC in promoting ester formation (*16*). It has been shown that the conjugate base of a urea is a reactive nucleophile toward a carboxylate anion (*17*). The potential dianionic intermediate thus generated can be avoided by concerted proton transfer from a Brønsted acid. This suggests that the conjugate base of biotin could be a good nucleophile toward sp^2 carbon centers of anionic species.



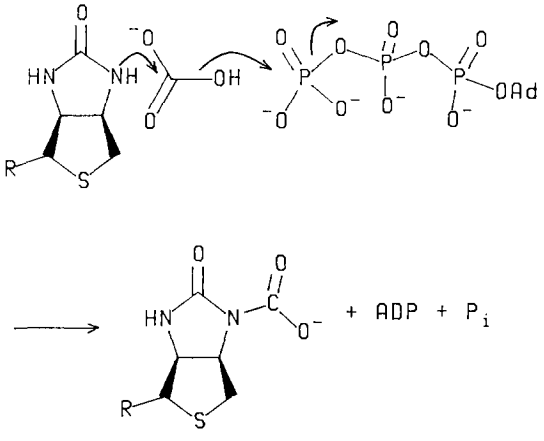
MECHANISTIC CONSIDERATIONS

Possible Mechanisms

These observations might provide a basis for inductively favoring a particular mechanism. But what mechanisms are possible? A systematic approach gives a basis for an evaluation. There are three reactants and three products. The order in which they react is unknown. To set out the possibilities, we analyze the reaction in terms of the molecularity of the step in which the bond between the ureido nitrogen of biotin and the carboxyl derived from bicarbonate is formed. While this is not necessarily the rate-determining step, it characterizes the various mechanisms, which are listed in terms of the paths through this process of the enzyme-bound species.

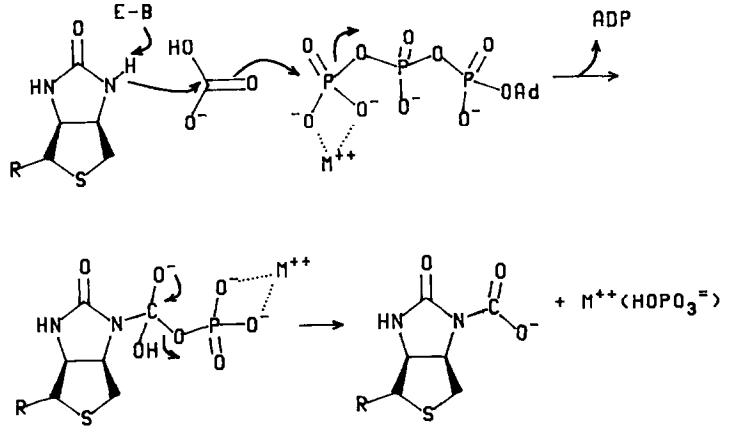
1. Termolecular Transition State (Biotin, Bicarbonate, ATP)

a. Direct formation of N-carboxybiotin. A concerted reaction involving ATP, biotin, and bicarbonate leads to a transition state containing all three species (*18, 19*). *This does not require that all three products are also present.* However, to our knowledge, the only published mechanisms with a trimolecular transition state show a completely concerted process. The mechanism, which was proposed in response to the discovery that oxygen is transferred from bicarbonate to ATP (*4*), involves a single step in which all the reactants assemble and all the products are formed (*18*). This means that there is direct displacement by the urea nitrogen center at bicarbonate in which hydroxide is expelled while attacking ATP. This is

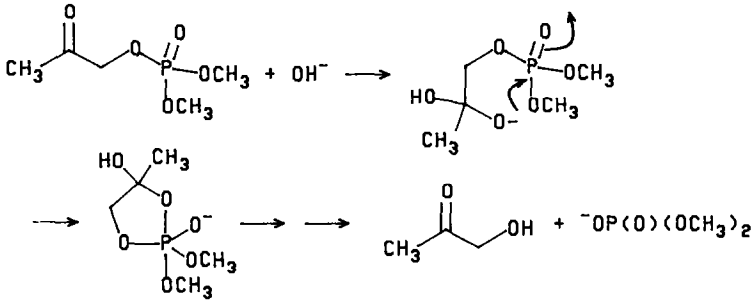


without chemical precedent or analogy since it involves the displacement of hydroxide from an sp^2 center. In addition, the role of ATP is not different from that of a proton (which would be better). However, these results are consistent with the observed absence of partial exchange reactions as well as the stereochemistry of the substitution process at phosphorus.

b. Trimolecular transition state (biotin, ATP, bicarbonate), producing an intermediate. Alternatively, the bond-forming step might generate an intermediate. The products are formed upon decomposition of the intermediate. In this mecha-



nism $N_{1'}$ of the conjugate base of the urea group adds to bicarbonate. The terminal phosphate of ATP traps the intermediate. The phosphorylated intermediate then expels phosphate to give the product. This requires that an incipient tetrahedral intermediate derived from the addition of the urea nitrogen of biotin to bicarbonate reacts as a nucleophile toward ATP. This type of reaction has precedent in the base catalyzed addition of a urea to a carboxylate (*17*). The urea nitrogen in the presence of base can function as a nucleophile toward a carboxylate and thus, by extension, toward bicarbonate. The reaction of the resulting tetrahedral intermediate with ATP is similar to the reaction of the conjugate base of a carbonyl hydrate with an adjacent phosphate triester (*19, 20*).



2. Bimolecular Reaction Forming an Intermediate That Reacts with the Third Component

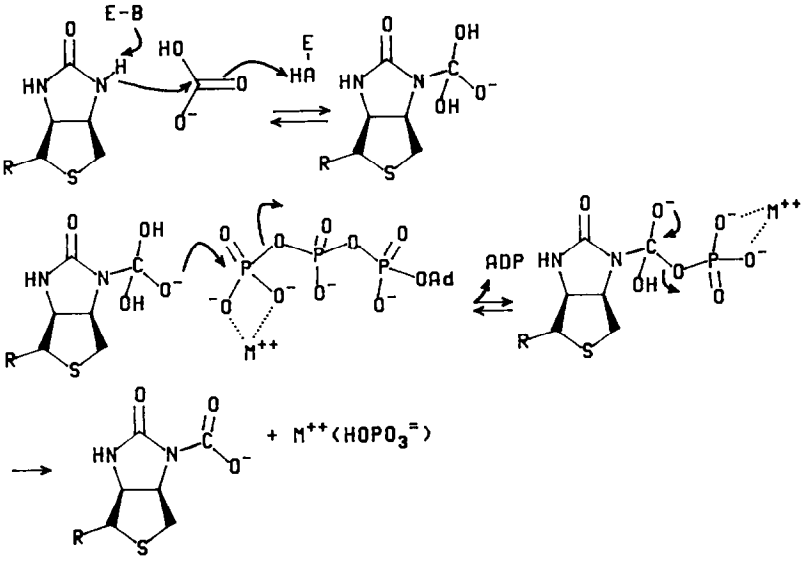
There are three possibilities in terms of the identity of the origins of the reacting partners. Each of these has further subdivisions in terms of mechanistic details. Most, but not all have been considered previously. All of these formally predict the existence of partial exchange reactions. The lack of such exchange reactions requires rationalization in terms of the requirements of product release steps (*3*).

a. (*Biotin + ATP*), *bicarbonate.* In this class of mechanism, the urea group of biotin becomes phosphorylated by ATP, generating ADP. The resulting phosphorylated intermediate reacts with bicarbonate at phosphorus and with the urea

nitrogen adding to the modified carboxyl derived from bicarbonate. Mechanisms involving phosphorylation either on oxygen of biotin (*14, 15, 18*) or on nitrogen (*21*) can be written and are consistent with model reactions. Stereochemical studies have placed restrictions on the possible steps of such a mechanism [(*8*), discussed below].

b. (*ATP + bicarbonate*), *biotin.* The most widely accepted mechanisms belong to this category. Bicarbonate reacts with ATP to produce carboxyphosphate which in turn serves as the carboxylating agent (*3, 10*). The reaction can first involve the formation of carbon dioxide which then reacts with the urea of biotin or the urea can attack directly (*11*).

c. (*Biotin + bicarbonate*), *ATP.* This is another possibility which has not yet been considered and is a variant of mechanism 1.b. In this case, the conjugate base of biotin reacts with bicarbonate to produce an addition intermediate which then reacts with ATP. It is likely that the phosphorus of the terminal group of ATP



would preassociate with an oxygen of bicarbonate. In particular, if the anionic center of bicarbonate associates with a cation, the π-electron density of bicarbonate would align with the phosphorus of the terminal phosphate of ATP. The addition of the conjugate base of a urea to a carboxylate (*17*) is an appropriate model for this mechanism. The intermediate should be very reactive toward ATP based on the observation that the conjugate base of a carbonyl hydrate reacts rapidly with an internal phosphate ester (*19*).

STEREOCHEMICAL CONSIDERATIONS

Hansen and Knowles reported that the stereochemical course at the phosphorus center which reacts is inversion of relative configuration (*8*). They propose

that this is best in accord with possibility 2.b and it is also in accord with a variant of 2.a which they do not support. It also is in accord with the two new mechanisms we have presented, 1.b and 2.c, since both involve a single displacement of the phosphate at phosphorus and such displacements proceed with inversion.

ATP as a Lewis Acid Catalyst

The systematic analysis we have presented leads to the consideration of two additional mechanisms for the enzyme-catalyzed carboxylation of biotin. The first involves the trapping of the tetrahedral intermediate derived from the addition of the urea of biotin to bicarbonate by ATP. The terminal phosphate group of ATP (which is coordinated to a metal ion) has considerable positive character at phosphorus and could serve as a Lewis acid catalyst for the addition. This arrangement could arise by preassociation of ATP as controlled by the enzyme. The phosphorus center should polarize the carboxyl group while the metal ion (or another electrophile) could neutralize the negative charges.

Relationship of Mechanisms to Carboxyphosphate

The intermediate generated in forming the C–N bond in mechanisms 1.b and 2.c is the phosphorylated adduct of biotin and bicarbonate. The same intermediate would be generated by the attack of biotin upon carboxyphosphate, a variant of 2.b in which carboxyphosphate reacts with biotin. While mechanisms related to 2.b have received considerable credibility, the existence of carboxyphosphate and its reaction patterns are problematic (*11*). The transfer of phosphate from ATP to bicarbonate may well do less to enhance the electrophilicity of bicarbonate than will coordination of the terminal phosphate of ATP to bicarbonate as a Lewis acid. This disrupts resonance stabilization of bicarbonate while formation of carboxyphosphate leads to a more localized resonance structure which is less susceptible to nucleophilic attack. The failure to trap carboxyphosphate or to detect partial exchange reactions (*3*) has made its existence in the reaction open to question. On the other hand, if the addition intermediate is generated directly, then the lack of exchange and the failure to trap carboxyphosphate are explained. The tetrahedral adduct generated from the addition of the urea anion to bicarbonate is expected to be a much stronger nucleophile than bicarbonate. Bicarbonate does not have to be generated from another source and it is a readily bound enzyme substrate. Carbon dioxide generated by the decomposition of carboxyphosphate would be a better electrophile but it would not be bound and would have to be trapped with 100% efficiency. Attempts to add carbon dioxide exogenously have not been successful (*9*).

Enzyme Catalysis: Other Participants

Perrin and Dwyer suggest that the intermediate formed by the addition of the conjugate base (at the urea moiety) of biotin to the carboxyl group derived from bicarbonate should be assisted by general acid catalysis (*12*) and this can be incorporated into the mechanism to avoid the high energy dianionic species. A base on the enzyme should be available to promote the ionization of biotin and

such a process would be kinetically competent. The metal ion requirements of the system indicate that there is a critical role for the metal (*3*). The interactions of anions with other anions as proposed here is hindered by electrostatics but the cationic metals can reverse the problem. A hypothetical role for a divalent metal is shown in the proposed schemes.

Resolution of Mechanistic Alternatives

Data that permit choosing among the mechanisms include the results of kinetic experiments that reveal the timing of the bond formation processes, reactions of analogs that unambiguously eliminate possibilities, trapping of intermediates, or the successful addition kinetically competent exogenously generated intermediates. Fitting data into the systematic framework permits a critical evaluation of the possibilities. Elucidating the details of catalysis and pathways within any one of these broad categories presents a further challenge.

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