CHAPTER 22

Dehydrogenation Mechanism in Flavoprotein Catalysis

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Introduction

The term "dehydrogenation" was originally used to describe a chemical process involving the abstraction of molecular hydrogen from an organic compound. Following Wieland's suggestion, it now has a broader use and means the rupture of (at least) one C—H bond, with concomitant transfer of its 2 electrons to a suitable acceptor:

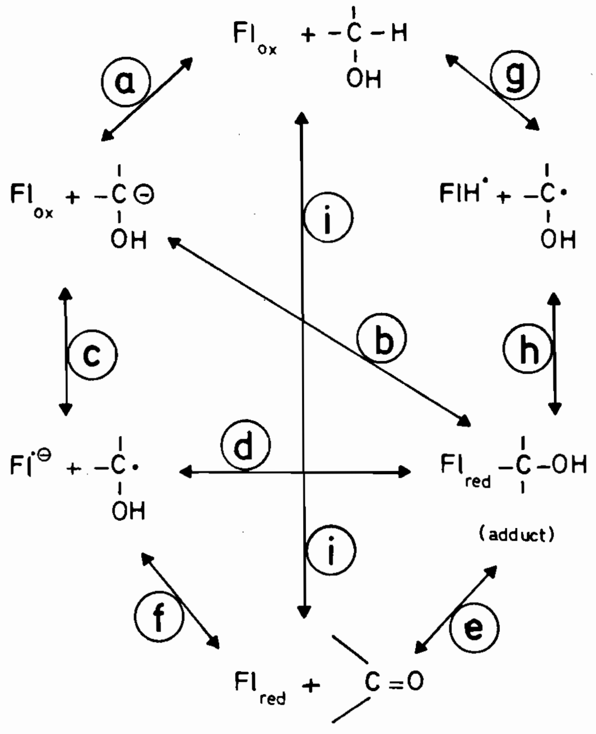
$$-\underset{\mathrm{H}}{\overset{|}{\underset{|}{\mathrm{C}}}}-\mathrm{X}-\mathrm{H}+\mathrm{Acc(ox)}\rightarrow \;\rangle\mathrm{C}=\mathrm{X}+\mathrm{Acc(red)H_2}$$

This type of reaction should be differentiated from the oxygenase reaction, in which the substrate electrons are transferred directly to oxygen. The limiting chemical step of the dehydrogenation, i.e., the rupture of the kinetically stable substrate C—H bond, is catalyzed by enzymes which have either pyridine nucleotide, pyridoxal-phosphate, flavin-, or pteridine as coenzymes.

The mechanism of the biological dehydrogenation step is undoubtedly a cardinal question and has been addressed since the discovery of redox enzymes. Michaelis' discovery of flavin radicals prompted the proposal that biological redox processes mandatorily proceed through single-electron transfer steps (1). This type of mechanism was revived in the early sixties by Beinert's discovery of stable flavoprotein radicals (2). Cornforth, however, was the first to make a concrete mechanistic suggestion proposing in 1959 that the oxidation of Acyl-CoA substrates by Acyl-CoA dehydrogenase is initiated by abstraction of the proton, and that it goes to completion by subsequent transfer of radical entities (3). In 1964, Hemmerich independently proposed a carbanion-initiated mechanism for the oxidation of "activated" substrates (4). It was not until the late 60's, however, that new inputs and ideas were put forward which constituted the stimulus for the progress that led to our present knowledge. This experimental work will be discussed below.

Definition of the Problem

Detailed mechanisms for the dehydrogenation reaction can be formulated as in Scheme 1.



Scheme 1. Alternative mechanisms for the transfer of redox equivalents between a "C—H" substrate and oxidized Flavin (Fl_{ox}). Further alternatives such as those initiated by $1e^-$ abstraction from the -OH group, and different modes of radical transfer subsequent to step g have been omitted for the sake of clarity.

They have been discussed elsewhere *in extenso* (5–11), and shall be summarized as "hydride mechanism" (step i), "radical mechanism" (g and following ones) or "carbanion mechanism" (step a). A dehydrogenation reaction initiated by step a can proceed via a covalent intermediate and its fragmentation (steps b+e) or by transfer of a single electron (c) to form a radical pair. The latter can then either collapse to a covalent adduct (d) or transfer a further radical to form the products (f). As discussed elsewhere, an experimental differentiation between steps b and c+d might be impossible, since such reactions are very fast (10, 11). The relevant differentiation between sequences c+f and b+e might, however, be possible as discussed below.

Chemical Work

The recognition of the flavin positions 4a and 5 as the most chemically reactive functions that are also likely to be involved in catalysis, and the investigation of the different reduced flavin isomers, including their mode of formation, is

the principal merit of extensive work by Hemmerich's group (see reference 2 for a review). Hamilton's proposal (12) that flavin catalysis is initiated by addition of the -X function of a R_2-CHXH substrate to the isoalloxazine 4a position is based on poor experimental evidence and was later demonstrated to be untenable for theoretical reasons as well (7). However, this proposal did have the merit of stirring up discussion and prompted fruitful experiments conceived to prove it wrong. On the other hand, work initiated by Weatherby and Carr (13), then completed and extended by Bruice's group (7, 14), and also results by Rynd and Gibian (15) provided the first evidence that carbanion species can chemically reduce flavin models. An extensive and comprehensive work by Bruice et al. on the interaction of "C—H acids" with flavin models was reviewed recently (7, 10). The most relevant conclusion from this work is that deprotonation, i.e., step a, Scheme 1, is chemically feasible in those instances where the electron pair of the carbanion can be delocalized and precedes Fl_{ox} reduction. With respect to the alternative modes of redox transfer from carbanion to Fl_{ox}, Bruice concludes that it might proceed via radical intermediates [c+f, Scheme 1; (10)]. While this deduction is not being questioned for a free chemical system, where specifically modified substrate and coenzyme models have to be used, within an enzyme active site different reaction profiles can be preferred, as will be discussed below. Also, in organic systems, transfer occurring via covalent adducts has been reported (16). Experiments involving addition of sulfite and phosphines (17, 18), or photosubstrates (6), and theoretical calculations (19) concur in predicting that the N(5) position is the one involved in transfer of redox equivalents:

$$Fl_{ox} + |X_{red} \longrightarrow Fl_{red} + X_{ox}$$

Enzymatic Work

In the early 70's, a new chapter of flavin enzymology was initiated by a series of papers dealing particularly with molecular mechanisms: Tober et al. (20) demonstrated that succinate dehydrogenase catalyzes the elimination of F^- from 2,2-difluorosuccinate, a reaction likely to be initiated by abstraction of the relatively acidic substrate α-proton. Shortly thereafter, in 1971, Miyake et al. (21) reported at an "ISOX" conference that D-amino-acid oxidase processes catalytically the substrate along β-Cl-alanine. In the discussion of these results, the formation of pyruvate as a product was mentioned; however, the crucial deduction that this might occur via abstraction of the α-H as a proton, and Cl^- elimination to form the enamine was not put forward. The merit of recognizing such a mechanism thus goes to Walsh et al. (22). In their elegant work, elimination of Cl^- from β-Cl-alanine was demonstrated to be a process indeed catalyzed by D-amino acid oxidase. This work received the title "Evidence for Removal of Substrate Hydrogen as a Proton" and was interpret-

ed as direct evidence for a carbanion mechanism (cf. step a, Scheme 1). While it is not my intention to diminish the importance of this work, it should be stated, as has also been discussed elsewhere (11), and in particular by Bright (5), that these results are compatible with a carbanion mechanism but do not prove it. This is unfortunately taken for granted in some uncritical review articles and discussions which have recently appeared (6, 23). Later work by Abeles, Walsh, as well as by Massey's group, confirmed the ambiguity of such an interpretation (24). Yagi, in his review of D-amino acid oxidase reaction mechanism presents and discusses evidence in agreement with these conclusions (25).

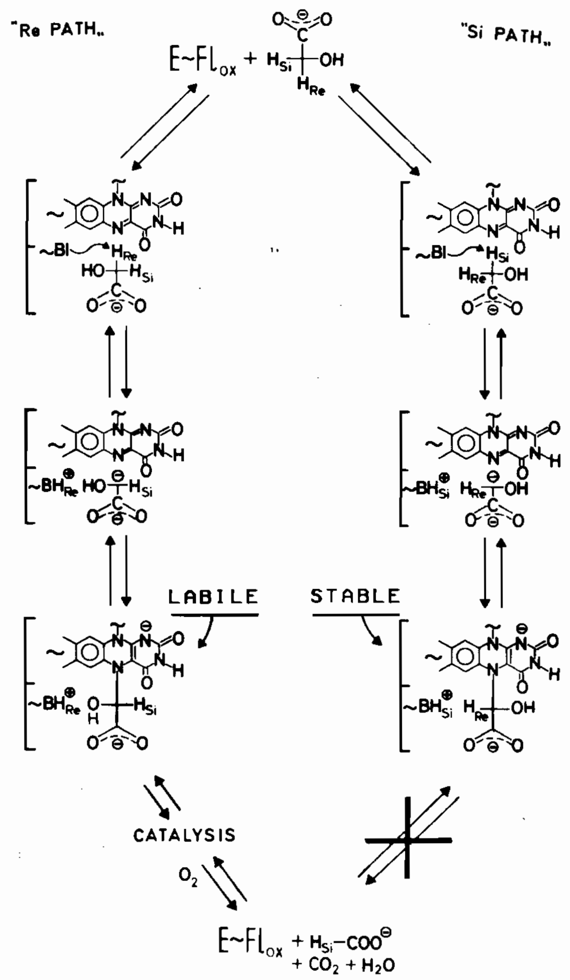
Of similar importance is the work by Bright's group involving the use of nitroalkane artificial substrates (26). These molecules are very efficient in the catalytic reduction of D-amino acid oxidase and glucose oxidase and in the presence of the CN^- as a trapping agent progressively and irreversibly inactivate the enzyme. Catalysis was thus proposed to proceed via covalent flavin N(5) adducts (5, 26) as shown:

Bruice, however, points out (7, 10) that nitroalkane anions are oxidized by a radical mechanism and that the formation of covalent adducts can be unique to the specific reactions with these enzymes.

Similar evidence for the involvement of position N(5) in catalysis comes from the "suicide" inactivation of flavin oxidases by acetylenic substrates, where in all cases formation of covalent adducts to N(5) was observed (see reference 27 for a review). This is also consistent with, but not direct proof for a carbanion mechanism.

Direct evidence for a carbanion intermediate and for covalent catalysis was obtained recently for the reaction of L-lactate oxidase and glycollate (11). From this alternate substrate, two glycollyl flavin N(5) adducts of different stability were formed and characterized. They are derived from chiral abstraction of either the Re or Si protons of the prochiral substrate via two parallel pathways (Scheme 2):

In the case of the stable adduct, which is derived from abstraction of the Si proton, it was shown that it arises directly from oxidized enzyme and glycollate and that it cannot be formed by alternate pathways ("Si path"). This is clearly incompatible with a hydride mechanism (step i, Scheme 1) and is evidence that a carbanion must initiate the reactions (see also Scheme 1, step a, and then either steps b or c). The companion, labile adduct is catalytically competent and occurs during abstraction of the substrate Re proton ("Re path"), i.e., it decays to the reduced enzyme glyoxylate complex which in turn reacts with oxygen to form the final products. Although in the second case a direct formation of the adduct from oxidized enzyme and glycollate cannot be obtained, it was concluded from the analogies between the "Si and Re



Scheme 2. This scheme represents the reactions of the prochiral substrate glycollate with L-lactate oxidase from *Mycobacterium smegmatis*. The left side ("Re path") represents the catalytic process proceeding via a labile flavin N(5) glycollyl adduct. The right side ("Si path") shows the dead end reaction leading to the stable diastereomeric adduct.

pathways" that the catalytic process proceeds by the same mechanism. The mechanism of Scheme 2 is in agreement with the work and proposals of Lederer's group concerning the flavocytochrome b_2 (28).

Evidence in favor of a carbanion mechanism was also obtained recently with acyl-CoA dehydrogenases and glutaryl-CoA dehydrogenase by Gomes et al. (29). With the first, enzyme inactivation by acetylenic inhibitors does not involve flavin reduction and is proposed to proceed via the corresponding allene intermediate:

$$-C{\equiv}C{-}CH(H\cdots B\sim){-}CO{-} \longrightarrow -C(H){=}C{=}CH{-}CO{-} \longrightarrow -C(H){=}C(B){-}CH_2{-}CO{-}$$

Similarly, Wenz et al. (30) have shown that 3,4-pentadienoyl-CoA is tautomerized to the 2.4 analogue by general acyl CoA dehydrogenase, possibly over a flavin covalent intermediate:

$$\left[\begin{array}{l} Fl_{ox} \quad B \\ H_2C{=}C{=}CH{-}CH(H){-}CO{-} \end{array}\right] \longrightarrow \left[\begin{array}{l} N{-}N^{\ominus} \quad HB^{\oplus} \\ HC{=}C{-}CH{-}CO{-} \\ \quad HC \; ? \end{array}\right] \longrightarrow \left[\begin{array}{l} Fl_{ox} \quad B \\ H_2C{=}CH{-}HC{=}CH{-}CO{-} \end{array}\right]$$

Is a Carbanion Transient Energetically Feasible?

Several authors have questioned the feasibility of a carbanion transient state from thermodynamic considerations, in particular by suggesting that the developing negative charge cannot be stabilized by delocalization (23). These claims can be disputed (10), and although they cannot be discussed in detail here, it should suffice to point out that nature has created several enzyme types that work by the carbanion mechanism (31). The pK of a weak acid such as C—H of lactate can be estimated to be around 25–30 (32). With the enzyme L-lactate oxidase, binding of transition state analogs such as dianionic oxalate cause the pK of an enzyme base to be increased by 7 pK units (33). Clearly, at the enzyme active center and with a properly fitting substrate, the microscopic pK of a C—H bond in question could be lowered by at least the same amount, thus bringing it into the range accessible for catalysis (33).

The Deazaflavin Dilemma

5-Deazaflavin have been used as coenzyme analogs of most classes of flavin enzymes (6,8,34). Four sets of experiments are directly pertinent to the question of flavoprotein dehydrogenation: (a) The reaction of enzyme-bound 5-deazaflavins with reduced pyridine nucleotides leads in incorporation of the 4-H of the latter into position C(5) of the coenzyme analogue (35); (b) The same is the case with the α-H of lactate or of amino acids (35,36); (c) No elimination of halide from β-halogenated substrates is observed with 5-deaza enzymes (35); (d) No covalent adducts were observed in the cases where reduction occurred (35–37) and no inactivation (and covalent adduct formation) was observed with α-hydroxybutynoic acid (37). Also, no reduction or adduct formation was observed with glycollate and 5-deaza FMN lactate oxidase (35).

These results clearly can be taken as apparent evidence for a hydride, and against a carbanion mechanism, and contrast sharply with the procarbanion evidence discussed above. As has also been suggested by Fisher et al. and by Pompon and Lederer (35, 37), there are escapes from the dilemma: according to Hemmerich (6) deazaflavins are pyridine nucleotide rather than flavin analogues and might thus work by a hydride mechanism. Walsh's group has provided some evidence that covalent adducts of deazaflavins might not be as stable as one would expect (35). The transfer of redox equivalents from a carbanion to deazaflavins might be unique for the latter and proceed by an alternate mechanism involving sequential transfer of electrons and protons (35). Thus, in spite of the wealth of experiments carried out with deazaflavins, uncertainty remains. On the other hand, at least one point emerges: namely, that the differences between this and the native coenzyme are such that direct mechanistic deductions cannot be extrapolated from one case to the other (6).

Comparison of Substrate Reactivities Within Different Types of Enzymes

If one assumes that pyridine nucleotide-dependent enzymes work by a hydride and pyridoxal phosphate ones by a carbanion mechanism, then the reactivity of flavin enzymes with the same (type of) substrates or pseudo-substrates can be used as a suitable mode of comparison (Scheme 3).

Thus pyridoxal phosphate enzymes catalyze eliminations and can be inactivated by acetylenic or comparable inhibitors (31, 39), while the same types of reactions have never been observed within pyridine-nucleotide enzymes. This places flavin enzymes unequivocally in the same "activation" category as pyridoxal phosphate-dependent ones, a strong argument in favor of a carbanion mechanism for both.

Conclusions

An objective interpretation of the results discussed above leads to the conclusion that a direct and definitive proof of a carbanion mechanism does not as yet exist. The sum of the evidence is, however, most clearly in its favor. With respect to the further course of the reaction, a mechanistic differentiation reduces to the problem of definition of the sequence of steps such as those defined in Scheme 1. A kinetic approach is bound to be of very difficult

COMPARISON OF SUBSTRATE REACTIVITIES WITH ENZYMES CATALYZING RUPTURE OF THE C-H BOND

SUBSTRATE TYPE	PYRIDOXAL	FLAVIN	NICOTINAMIDE
$Y-CH_2-C(H)(XH)-$ Y = leaving group X = CH, NH_2	$Y-CH_2-C^{\ominus}(-\overset{+}{X}H)-$ ↓ $Y^{\ominus}$ + $H_2C=C(XH)-$ (elimination)	$Y-CH_2-C^{\ominus}(XH)-$ ← →	$Y-CH_2-C(H)(XH)-$ ↓ $Y-CH_2-C(=X)-$ (oxidation)
$H-C\equiv C-C(H)(X)-$ X = CH, NH_2	$H-C\equiv C-C^{\ominus}(XH)-$ ↓ $H_2C=C=C(XH)-$ (inactivation)	$H-C\equiv C-C^{\ominus}(XH)-$ ← →	$H-C\equiv C-C(H)(XH)-$ ↓ $H-C\equiv C-C(=X)-$ (oxidation)

Scheme 3. Comparison of reactions catalyzed by different classes of enzymes with the same type of substrate.

experimental verification for the reasons outlined above and elsewhere (11). On the other hand, a thermodynamic approach comparing the reaction profiles of the reaction paths involving covalent intermediates (Scheme 1,b,e), with those proceeding via radical transfer (c,f) might give a clue: such a comparison was made by Williams and Bruice for the chemical system, and suggested a radical transfer sequence (32). In the case of flavin-dependent dehydrogenase reactions, where reliable data exist, e.g., with glucose oxidase, it appears that radical stabilization results from kinetic as well as from thermodynamic factors (40). In the cases of methanol oxidase (41,42), and bacterial luciferase (43), an extremely strong radical stabilization was observed; with these enzymes, however, a catalytic role for these radicals is excluded. In other cases, e.g., lactate oxidase (44), and acyl CoA dehydrogenase (this volume), a thermodynamic stabilization of the radical is at least possible. However, no clear-cut example of flavin radical involvement in catalysis has ever been reported for this class of enzymes. On the other hand, a clear case of covalent intermediate stabilization has been documented with L-lactate oxidase, where the catalytically competent N(5) covalent adduct is in a ~1:1 (rapid) equilibrium with the products (45). Thus, even if at present the experimental evidence is in favor of covalent catalysis, I feel that a generalization cannot yet be made, radical transfer being a reasonable alternative.

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