ABSTRACT: Flavins are versatile biological cofactors which catalyze proton-coupled electron transfers (PCET) with varying number and coupling of electrons. Flavin-mediated oxidations of nicotinamide adenine dinucleotide (NADH) and of succinate, initial redox reactions in cellular respiration, were examined here with multiconfigurational quantum chemical calculations and a simple analysis of the wave function proposed to quantify electron transfer along the proton reaction coordinate. The mechanism of NADH oxidation is a prototypical hydride transfer, with two electrons moving concerted with the proton to the same acceptor group. However, succinate oxidation depends on the elimination step and can proceed through the transfer of a hydride or hydrogen atom, with proton and electrons moving to different groups in both cases. These results help to determine the mechanism of fundamental but still debated biochemical reactions and illustrate a new diagnostic tool for electron transfer that can be useful to characterize a broad class of PCET processes.

INTRODUCTION

Enzymes equipped with flavin cofactors comprise the most abundant class of natural catalysts for combined proton and electron transfer.1,2 The redox center in all natural flavins is formed by the heteronuclear tricylic isalloxazine ring (Figure 1), primarily attached to the protein by noncovalent hydrogen bonds, stacking, and cation−π contacts.3−5 These interactions also modulate the flavin redox potential from −400 to 60 mV, allowing oxidation of a range of aliphatic and aromatic substrates.6−8

Flavin redox reactions are an example of proton-coupled electron transfers or PCET, a broad family of reactions and energy conversion processes in chemistry.9−11 PCET mechanisms are characterized by the number of electrons involved, such as in hydride (2e−/1H+) versus hydrogen-atom (1e−/1H+, or HAT) transfer;12 the order of steps or their concurrency, such as electron transfer first, proton second (ETPT), or concerted proton−electron transfer (CPET);13 whether the transfers proceed from/to the same or different chemical groups in the donor(acceptor), as in multiple-site PCET;10,12 and the tunneling behavior and the adiabacity or participation of excited-states in the transfer processes.13,15

Thermochemical and redox potential measurements determined that flavins may undergo hydride and single-electron (1e−) transfers,10,16 and molecular simulations explored their tunneling effects.17−20 However, a description of possible HAT, particularly in enzymatic mechanisms, has received less attention.21 A remarkable example is the BLUF flavoprotein which has been shown by ultrafast spectroscopy and simulations to sustain HAT by light activation.22,23

Quantitative diagnostics of PCET mechanisms can be obtained from quantum-chemical calculations of molecular properties along the reaction progress (usually the proton transfer coordinate).13 Nonadiabatic couplings15 and dipole moments24 are well-behaved properties. However, the calculation of partial charges and spins,25 the simplest and most common diagnostic used,13,26−28 can be problematic because these are not physical observables and may be calculated with different recipes.29−31 Realizing these difficulties, intrinsic bond orbitals32 were recently proposed and successfully applied to identify proton and electron donors in PCET.33,34

Here, we address the PCET mechanism in flavin-mediated oxidation of NADH (reduced nicotinamide adenine dinucleotide) and of succinate catalyzed respectively by respiratory complex I (or type I NADH dehydrogenase)35−37 and by complex II (or succinate dehydrogenase, see Figure 1).38,39 These fundamental steps for cellular respiration have been traditionally assigned to hydride transfers,36,39 but recent...
Optimizations with methods neglecting dispersion interactions residues and with previous calculations on similar reactions.40 E1cb elimination from H2Suc (butanedioic acid) and conjugate base elimination (E1cb) in step (c). Biologically relevant flavins differ only in the R1 substituent. R2 is the ribosyl-ADP moiety, and B− is a general base.

These models had 45, 44, and 51 atoms, respectively.

Figure 1. Flavins mediate oxidation of NADH and succinate. NADH reacts by elimination from the nicotinamide ring, step (a). Two reaction sequences are possible for succinate oxidation: unimolecular elimination (E1) is shown in step (b) and conjugate base elimination (E1cb) in step (c). Biologically relevant flavins differ only in the R1 substituent. R2 is the ribosyl-ADP moiety, and B− is a general base.

The complete active space self-consistent field (CASSCF)28,41 method was used with the PySCF package version 1.548 for singlet spin states in C1 point-group symmetry. All single-point calculations used the def2-TZVP basis set. Configuration and population analysis on CASSCF wave functions were done with localized orbitals by the Pipek–Mezey scheme, while Foster–Boys localization led to equivalent results.49 This analysis was also performed on CASSCF/def2-SVP wave functions, and similar results were observed. Active spaces contained six electrons in five MOs (6e,5o) for H2Suc, (8e,6o) for H2Suc−, and (12e,10o) for MNAH reactions as determined from analysis of occupation numbers and selection of reactive MOs. As MOs may change shape and ordering along the reaction path, CASSCF calculations started from the geometry with highest multiconfigurational character (product for H2Suc and reactant for H2Suc− and MNAH reactions), and guess MOs were taken from the CASSCF calculation of an adjacent geometry along the path.

The short-range influence of the enzymatic environment on the mechanism of flavin-mediated oxidation was emulated with truncated active site models (Figure S1, Supporting Information). For succinate oxidation catalyzed by respiratory complex II, the model contained 192 atoms including FAD (for which the ribityl-ADP moiety was replaced by 2-hydroxyethyl), succinate (deprotonated, Suc), side chains of H365, T377, E378, R402, H504, and R544, backbones of G183 and E184, methyl groups, side chains of residues N92, E97, Y180, N220, S295, and T325, and backbone of residues H136, T138, and M139.

Figure 2. The reaction profile was obtained with constrained geometry optimizations fixing r1 while relaxing all others degrees of freedom using the ORCA 4.1.1 program.46 Intrinsic bond orbitals (IBOs)35 were obtained from localization33 of the broken-symmetry solution34 of unrestricted M06 single-point calculations with ORCA.

The model contained 167 atoms including FMN, NADH (for which phosphate group and ADP moiety were replaced by methyl groups), side chains of residues N92, E97, Y180, N220, S295, and T325, and backbone of residues G183 and E184 from the Nqo1 subunit of T. thermophilus complex I as found in the PDB ID 3IAM35 structure. This model corresponds to the MNAH reaction in Figure 2A. Geometries of stationary

confirmed by analysis of the Hessian eigenvector with the negative eigenvalue and of the intrinsic reaction coordinates (Table S1, Supporting Information).35
points were optimized in the B3LYP-D3/def2-SVP level and kindly provided by Prof. Ville Kaila from their original study. Geometries for the complex II model were optimized at the M06-L/def2-SVP level. Geometries of the enzyme models showed little dependency on the optimization level if dispersion interactions were accounted for (as in B3LYP-D3 or M06-L functionals). Wave functions for stationary points were obtained at the CASSCF/def2-SVP level with the same active spaces used for the isolated reactions.

## RESULTS AND DISCUSSION

Three model reactions corresponding to steps (a)–(c) in Figure 1 were studied with a lumiflavin (LF, R1 = methyl) acceptor, NADH modeled as 1-methylnicotinamide (MNAH), and succinate protonated to H2Suc (succinic acid) for E1 elimination and H2Suc\(^-\) (carbanion ↔ enolate electromer) for E1cb elimination. See panels A, B, and C in Figure 2 for the reactant structures, consistent with enzymatic active sites. In order to quantify the extent of electron transfer along the reaction and avoid the pitfalls of using only partial charges to analyze PCET mechanisms, we propose a simple quantitative diagnostic of electron transfer employing weights of wave function configurations constructed with orbitals localized in the donor. This electronic charge transferred, \(\Delta\text{oxx}(\text{RC})\), is defined along the reaction coordinate (RC) as

\[
\Delta\text{oxx}(\text{RC}) = \sum_n n \omega_n(\text{RC}) - \max\{n\}
\]

where \(n\) is the number of electrons occupying active MOs localized in the donor, \(\max\{n\}\) is their maximum occupation at the reactant state, and \(\omega_n\) is the combined weight of wave function configurations with \(n\)-electrons. Only configurations with \(\omega_n > 1\%\) in at least one geometry along the reaction were considered. This analysis may be used with any kind of multiconfigurational wave function obtained with localized MOs.

We start with the H2Suc reaction (model for succinate E1 elimination, Figure 2B) in which the broken C–H bond \(\sigma\) MO becomes a nonbonding MO in the product. This is the only active MO localized in the H2Suc donor, thus \(\{n\} = \{0, 1, 2\}\) electrons and \(\max\{n\} = 2\). Figure 2B middle column shows that the contribution (combined weight) to the wave function of this localized MO changes from almost 100% for two-electron occupation in the reactant to 80% for one-electron occupation in the product. The average electronic charge transferred is \(\Delta\text{oxx} = -1\), showing that the H2Suc reaction is a hydrogen-atom transfer (HAT).

In the biradical product, an unpaired electron occupies the LF \(\pi^*\) MO and spin-couples to a singlet state with the unpaired electron in the carbocation (Figure 2B, top of middle column), resulting in a short distance (\(r = 2.3\) Å) between the two molecules. Interestingly, this is in line with experimental...
observation of a FAD flavosemiquinone radical signal during the catalytic cycle of respiratory complex II.53

Partial charges condensed on the LF and H2Suc groups are similar (within 0.2 e, Figure 2B, right column) and nearly neutral when comparing reactant and product (end) states, suggesting there is no net charge separation or redistribution, in accordance to a HAT.13,15 The partial charge on the transferred H atom is close to 0.2 e along the complete pathway for all reactions studied here (Figure 2, right column). A naive interpretation of this partial charge would suggest a HAT is observed in all reactions, but this is not the case as clearly shown for the other two reactions below.

For H2Suc (E1cb elimination, Figure 2C), the nonbonding MO with the extra electron lone-pair and the σ MO of the broken C−H bond localize in the donor. Only configurations with \( \{n\} = (2, 3, 4) \) electrons in these orbitals contribute significantly (more than 1%) to the wave function, thus \( \max\{n\} = 4 \). Remarkably, the anionic reactant already shows a substantial charge transfer, \( \Delta \text{oxi} = -0.5 \) at RC = -1.0 Å. When fumaric acid is formed, only configurations with two electrons in the forming double bond become relevant, resulting in a much longer distance (\( r_1 = 3.8 \) Å) between LF− and fumaric acid. The average \( \Delta \text{oxi} = -2 \) in the product, and this reaction is clearly a hydride transfer. Partial charges change considerably between end states, and LF has a −1e charge in the product, as expected from receiving a hydride.

For the MNAH reaction (Figure 2A), the MO of the broken C−H bond becomes a π MO and conjugates with the other nicotinamide MOs. A total of six active MOs are localized in the donor. Only configurations with \( \{n\} = (6, 7, 8) \) electrons in these orbitals contribute significantly to the wave function, thus \( \max\{n\} = 8 \). Weights change from almost 100% for configurations with eight electrons in the reactant state to 85% for six electrons in the product, with a final \( \Delta \text{oxi} \approx -2 \). We conclude this reaction is also a hydride transfer.

It is noteworthy that weights for configurations with an odd number of electrons localized in the donor (one electron for H2Suc, three for H2Suc−, and seven for MNAH reactions) peak near the transition state (TS, RC = -0.03 Å for MNAH, 0.61 Å for H2Suc, and 0.14 Å for H2Suc−, Table S1). This is in line with \( \Delta \text{oxi} \approx -1 \) at the TS for the three reactions (Figure 2, middle column) and suggests that the activation energies correspond to the first (and only in H2Suc reaction) electron transfer. Partial charge or other population analysis10−32 cannot usually provide this level of detail.

Electron and proton transfer occur concerted along the same range of reaction coordinates for the three studied reactions (H transfer indicated in Figure 2). However, analysis of intrinsic bond orbitals (IBO,32,33 Figure 3A) suggests that the

![Figure 3.](https://dx.doi.org/10.1021/acs.jcim.0c00945)
two transfers take place from (or to) the same donor (acceptor) site only for the MNAH reaction as orbitals (and the corresponding electron density) from the C−H bond in the reactant turn into the N5−H bond in the product after H− transfer. On the other hand, electron and proton transfer in the two succinic acid reactions proceed to different sites of the acceptor flavin. These two reactions should be classified as concerted PCET\(^{11,13}\) or multiple-site PCET\(^{14}\) as orbitals of the broken C−H bond in the reactant do not turn into the N5−H bond in the product. For the \(\text{H}_2\text{Succ}\) reaction, the one electron transferred from the broken C−H bond to the flavin delocalizes over the pyrimidinedione ring (Figure 3B). For the \(\text{H}_2\text{Succ}^+\) reaction, the broken C−H bond rearranges to form the double bond in fumaric acid (Figure 3C), and the two electrons transferred to flavin come from the nonbonding MO with the extra electron lone-pair (Figure S2).

Finally, we tested whether the mechanisms observed above are conserved in the enzymatic environment. Truncated active site models with 167 and 192 atoms, respectively, coordinating NADH in respiratory complex I and succinate in respiratory complex II were built from these protein crystallographic structures (Figure S1).\(^{55,57,38}\) Analysis of \(\Delta\alpha\)ν (eq 1) calculated for these active site models using the same methods depicted above shows NADH oxidation proceeds via hydride transfer, and succinate oxidation in E1 elimination occurs through HAT (Table S2), showing that the enzymatic mechanisms are equivalent to those of the isolated models.

### Conclusions

We confirm the traditional view\(^{36}\) that NADH oxidation by flavin proceeds via a prototypical hydride transfer, with the two electrons moving concerted with the proton from the donor to the same acceptor group. A previous proposal of HAT for this reaction\(^{27}\) is incorrect and illustrates the pitfalls of assigning PCET mechanisms with diagnostics based only on partial charges. For succinate oxidation by flavin, two reaction sequences are possible. E1 elimination may occur by a HAT, opposed to usual proposals,\(^{39}\) but E1cb elimination will also proceed via hydride with an advanced charge transfer in the reactant state.

PCET mechanisms of flavin-mediated oxidation depend on the donor molecule. This should have implications for the mechanisms of several flavoproteins besides those of the respiratory chain studied here. It is also expected that the simple diagnostic of electron transfer along the reaction pathway proposed here will be useful to characterize a broad class of PCET processes.

### Associated Content

#### Supporting Information

The Supporting Information is available free of charge at https://pubs.acs.org/doi/10.1021/acs.jcim.0c00945.

Two figures with the structure of truncated enzyme active site models and additional IBO analysis and two tables with quantification of electron transfer (\(\Delta\alpha\)ν) for enzyme models and TS analysis.\(^{PDF}\)

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### References


FMO and QM/MM Methods.


