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Characterization of the Transition State for the Hydride Transfer in a Model of the Flavoprotein Reductase Class of Enzymes

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The transition state structure for the hydride transfer between 1,4-dihydronicotinamide and the flavin isoalloxazine moiety has been characterized by means of the AM1 and PM3 semiempirical methods. The distance between the initially H-bonded carbon atom of 1,4-dihydronicotinamide and the nitrogen atom of the flavin isoalloxazine moiety is found to be 2.67 Å with both methods. The transferring hydrogen is located closer to the nitrogen atom than to the carbon atom, with an almost linear arrangement. Finally, an insight into the electronic nature of the transfer is obtained from Mulliken atomic charge population and Bader analyses: the former shows an atomic charge of 0.18 a.u. for the formally termed *hydride*, while from the latter, bond critical points for the breaking (C4′–H) and forming (H–N5) bonds have been located and electronic charge density and laplacian contour maps have been built. Finally, mechanistic implications of the overall results are discussed.

INTRODUCTION

Flavoprotein reductases are a family of enzymes that includes glutathione reductase (*1*), lipoamide dehydrogenase (*2*), thioredoxin reductase (*3*), asparagusate dehydrogenase (*4*), trypanothione reductase (*5*), and mercuric ion reductase (*6*), among others. At present, far more than 100 different flavoenzymes are known, most of them members of the class of oxidoreductases (*7*). Despite the large variety of this family, it has been found that they are structurally and mechanistically related.

To date, the only disulfide oxidoreductase whose X-ray crystal structure has been solved to high resolution (1.5 Å) is human glutathione reductase (GTR) (*1*). This is the reason why this enzyme has been usually taken as a reference for the rest of the family. Common aspects between this enzyme and others have been explicitly found. In particular, lipoamide dehydrogenase is very similar to GTR both in structural and mechanistic aspects (*8*). Likewise, the bacterial enzyme mercuric reductase has a great resemblance to GTR (*9*). All members of this family share also similar spectral properties (including the diagnostic flavin–thiolate charge-transfer band) and high active site sequence homology.

On the other hand, the reduction of enzyme-bound flavin cofactors by reduced pyridine nucleotide analogues is an important reaction in biochemistry (*10*). From

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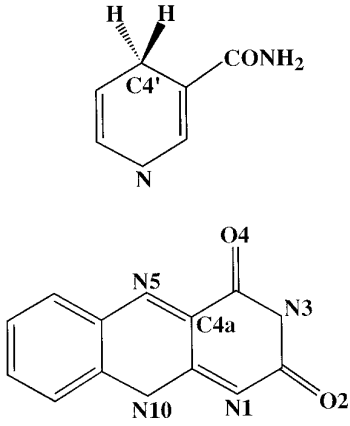
a mechanistic point of view, the transfer of a hydride equivalent involves six possible mechanisms (*11*), which, at the same time, can be gathered into three different groups depending on the nature of the migrating H: (1) transfer of a *hydride* ion in a single step; (2) two-step transfer of an electron before or after the transfer of a *hydrogen* atom; and (3) transfer of two electrons and a *proton* in three separate steps and orders. Furthermore, even considering the hypothesis of a concerted one-step pathway, the coupling between the H motion and the electronic shift has to be kept in mind (*12*). The overall details on merged mechanisms for hydride transfer from 1,4-dihydronicotinamides can be found in a recent review by Bunting (*13*). Furthermore, a theoretical framework for the partial uncoupling of the electron transfer and hydrogen migration events can be nicely obtained from the valence-bond configuration mixing model developed by Shaik and Pross (*14*).

Focusing our attention on the reductive reaction, a common mechanism has been proposed for many flavoenzymes, involving the transfer of the equivalent of a hydride ion from C4′ of NADH to N5 of the flavin isoalloxazine ring (FAD):

$$\mathrm{NADH + FAD \rightleftharpoons [NAD \cdots H \cdots FAD]^{\neq} \rightleftharpoons NAD^{+} + FADH^{-}}.$$

However, not only the mechanistic aspects of this reaction but also the electronic nature of the formally termed *hydride* being transferred is still a matter of controversy. For instance, Suelter and Metzler (*15*) studied the nonenzymatic reduction of several flavin analogues by 1-propyl-1,4-dihydronicotinamide and concluded that a hydride transfer was taking place. The reaction mechanism for net hydride transfer from dihydronicotinamide to flavin was later formulated by Bruice *et al.* (*16*) to involve preequilibrium complex formation along the reaction path. Blankenhorn (*17*) proposed the formation of flavin–nicotinamide charge-transfer complexes as catalysts in the transfer of a hydride ion between dihydronicotinamide and flavin and stated that their catalytic function would consist in providing preorientation of the reactants in a geometry similar to that of the transition state, leading to prepolarization of the C4′–H dihydronicotinamide bond, to be broken in the rate-limiting step of the reaction. Powell and Bruice (*18*) performed a comparison between a hydride ion and electron transfers in the reduction of flavin and flavin radical by 1,4-dihydronicotinamides, concluding that a direct hydride transfer could be the most favorable mechanism. More information on the structural nature of these complexes was furnished by an exhaustive study on the absolute stereochemistry of flavins in enzyme-catalyzed reactions (*19*), which showed that all tested flavoenzymes interact via the *re* face of the flavin ring with the substrates used. More recently, in order to provide more information on the evolution of the hydride transfer process along the reaction, several experimental attempts have been made trying to elucidate the nature of the transition state for this reaction on glutathione reductase from kinetic isotope effects (*20*).

In this paper, we have investigated the structural, electronic, and mechanistic aspects of the previously mentioned hydride transfer using semiempirical quantum chemical calculations. In particular, special attention has been made to the characterization of the transition state and to changes undergone when going from the reactant complex to the product complex along the reaction path.



SCHEME 1

METHODOLOGY AND MODEL

The calculations were carried out using the AM1 (*21*) and PM3 (*22*) semiempirical methods. Transition states were fully optimized and characterized by diagonalizing the matrix of energy second derivatives to determine the unique imaginary frequency. In reactant and product complexes, optimizations were constrained so the distance between the C4′ of 1,4-dihydronicotinamide and the N5 of the isoalloxazine moiety of FAD was fixed to 3.5 Å, according to X-ray cristallographic data (*1c*). A single point RHF/6-31G**//AM1 calculation was performed to test the reliability of the electronic analysis of the AM1 method, which considers only valence electrons. To build the intrinsic reaction path at the AM1 level, we used the reaction-path-following algorithm proposed by González and Schlegel (*23*), which requires only the first derivatives at points along the path in internal mass-weighted coordinates; this path is started from the transition state using a previously calculated numerical Hessian at the semiempirical level, to obtain the transition vector that follows downward. All calculations were performed by means of the Gaussian 92 program (*24*). Characterization of bond critical points and construction of charge density and laplacian maps were achieved by using the program Electra (*25*).

In these enzymes, in addition to the FAD, each active site contains a pair of cysteines which form a so-called redox-active disulfide. Although it is known that this disulfide is in close proximity to the flavin isoalloxazine ring to form a charge-transfer complex (*26*), it is believed that this complex is formed immediately after the hydride transfer has occurred, involving the rupture of the disulfide bond together with an electronic release to the nearby sulfur atom (*20b*, *27*, *28*). Since the main purpose of this contribution is to explicitly study the hydride transfer event, the presence of the disulfide was not considered. Thus, our model for a general flavoprotein reductase was constructed by taking the 1,4-dihydronicotinamide (a model for NADH) as hydride donor and the isoalloxazine moiety of FAD as hydride acceptor (Scheme 1). The atomic nomenclature used (C4′ of 1,4-dihydronicotinam-

ide and N5 and C4a of the flavin isoalloxazine ring) follows that of Schulz *et al.* (*29*) for FAD and that of Pai and Schulz (*1c*) for glutathione reductase.

RESULTS AND DISCUSSION

It has been previously stated (*16*, *17*, *27*) that the relative distance and position of the reaction partners NADH and FAD in the active site of flavoprotein reductases is oriented in such a way that they are prepared for the transition state structure along the reaction coordinate of the hydride transfer. This positioning eliminates the decrease in entropy required for this reaction and is in agreement with proposals concerning enzyme-catalyzed reactions in general. In addition, the complete proteinic environment around the active site of the enzyme will contribute to maintain these structural constraints.

For this reason, structures of the reactant (NADH–FAD) and product (NAD^+–$FADH^-$) complexes have been constrained to a C4′–N5 distance of 3.5 Å. The optimized structures of both complexes at the AM1 level are depicted in Fig. 1. From a structural point of view, the main noticeable result turns out to be the loss of planarity of the reduced isoalloxazine ring in the product complex, due to the fact that the central ring adopts a boat conformation. Such conformation is practically forced by the fact that, once the hydride transfer has been completed, the two reactant partners become charged and strong electrostatic interactions between them appear. Thus, the adoption of a boat conformation on the central ring of the reduced isoalloxazine part of FAD permits the lone pairs of the two nitrogen atoms in this ring to be directed toward the oxidized nicotinamide.

Another remarkable property of the structure adopted by the product complex is the fact that the transferred hydrogen on N5 is directed downward. As mentioned above, experimental evidence shows that, in a second step, $FADH^-$ transfers reduction equivalents to the redox-active disulfide located close to the C4a position of the isoalloxazine ring. After the formation of a covalent bond between C4a and the closest sulfur atom, a transfer of a proton from N5 to the sulfur of the other cysteine and fragmentation of the disulfide bridge should follow. The fact that the transferred hydrogen on N5 is correctly directed toward the pair of cysteines can be key evidence to support the aforementioned proposed mechanism after the hydride transfer takes place.

In Fig. 2 we have depicted the transition state structure fully optimized at the AM1 level. As indicated by a dashed line, a hydride is being transferred from the C4′ atom of the 1,4-dihydronicotinamide to the N5 atom of the flavin isoalloxazine ring. Table 1 may help to analyze the structural characteristics of this transition state at the AM1 and PM3 levels. One of the most relevant results is the C4′–N5 distance, which is, actually, the distance that must be travelled by the migrating hydride. The values obtained with the two semiempirical methods (2.67 Å) agree with those obtained for a series of theoretical studies on transition states of hydride transfers. For example, values of ca. 2.7 Å have been found for enzymic models of LADH (*30*), FDH (*31*), LDH (*32*), and DHFR (*33*). Thus, this distance seems to

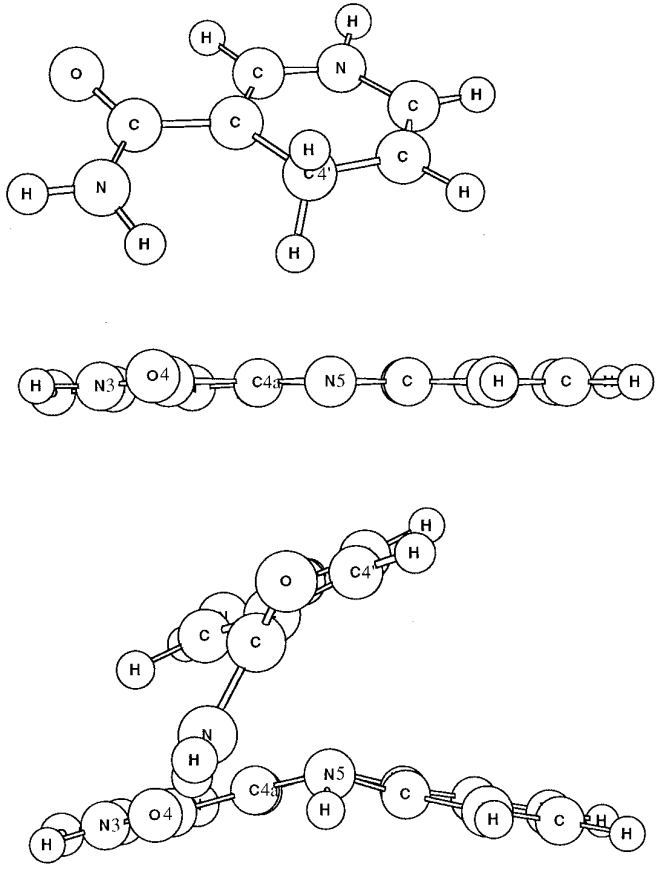


FIG. 1. AM1 optimized structures for the NADH–FAD reactant complex (top) and NAD^+–$FADH^-$ product complex (bottom), with the C4′–N5 distance constrained to 3.5 Å.

be a constant in the structure of transition states for hydride transfers, regardless of the nature of the hydride acceptor (*34*).

The other interesting structural result on the transition state is that the migrating H is clearly closer to N5 than to C4′, in an almost linear arrangement (Table 1). A systematic study on different model transition states for several enzymic hydride transfers (*34*) showed that once the 2.7 Å distance between C4′ and the acceptor heteroatom has been reached, the relative position of the H being transferred along this path depends on the capability of the hydride receptor to polarize the C4′–H bond and, from the Hammond postulate, on the relative stability of the reactant and product complexes. Moreover, the question of whether the three atoms involved in the hydride transfer adopt a bent or a linear arrangement in the transition state depends on the optimal frontier orbital interactions between the HOMO of NAD(P)H and the LUMO of the flavin isoalloxazine ring (*27*). In this case, using both semiempirical methods, it was found that the C4′–H–N5 atoms form an angle

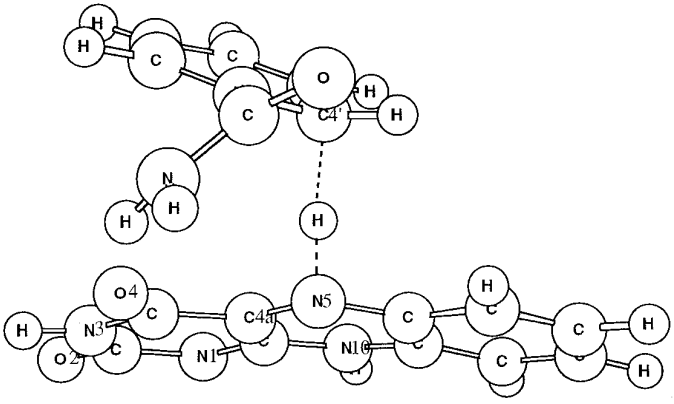


FIG. 2. AM1 optimized structure for the transition state.

of ca. 170° in the transition state, although it has been shown that semiempirical methods usually yield angle values larger than those of high level SCF *ab initio* calculations (*34*). Finally, we have added at the end of Table 1 the value of the unique imaginary frequency that characterizes the transition state.

In an attempt to obtain more information on how the overall hydride transfer process evolves from the reactant complex up through the transition state and finally down to the product complex, the intrinsic reaction path (IRP) from the transition state was constructed at the AM1 level. As shown in Fig. 3, the NADH–FAD reactant complex is much more stabilized than the NAD^+–$FADH^-$ product complex, the energetic barriers to the transition state being 37.68 and 27.74 kcal/mol, respectively; this fact is a consequence of the loss of planarity of the reduced isoalloxazine part. However, thinking about the influence of the solvent and the proteinic environment, the presence of polar interactions should decrease the reactant complex barrier to the transition state and increase the product complex barrier, thus, making both barriers more similar and helping the reversibility of the process.

TABLE 1

Structural Parameters (Distances in Å and Angles in Degrees) and the Unique Imaginary Frequency (in cm^{-1}) That Characterize the Transition State at the AM1 and PM3 Semiempirical Levels

Parameter	AM1	PM3
C4′–N5	2.672	2.667
C4′–H	1.480	1.492
N5–H	1.197	1.186
<C4′HN5	172.7	169.5
ν_i	−1701.3	−1763.2