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Transition structure and reactive complexes for hydride transfer in an isoalloxazine–nicotinamide complex. On the catalytic mechanism of glutathione reductase. An ab initio MO SCF study

W. Diaz [a], J.M. Aullo [a], M. Paulino [b], O. Tapia [b,*]

[a] *Department de Química Física, Universitat de València, Burjassot (València), Spain*
[b] *Department of Physical Chemistry, Uppsala University, Box 532, S-751 21 Uppsala, Sweden*

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Abstract

An analysis is presented of the catalytic mechanism of glutathione reductase based upon a theoretically characterized saddle point of index one obtained for a model representing the active groups of the flavine and nicotinamide adenine dinucleotide phosphate, namely, an isoalloxazine and nicotinamide rings. The isoalloxazine rings appears deformed into a butterfly conformation in the saddle point of index one. The butterfly conformation is retained along the path leading to a reduced isoalloxazine (N5–H) forcing the transferred hydrogen to stick into the nicotinamide binding site, this geometric feature suggests the existence of a transposed hydride transfer path where the N5-proton goes back to a lysine residue leaving the electrons on FAD. This mechanism is discussed and proposed as an alternative catalytic pathway in glutathione reductase to the standard electron transfer one. These results help to understand the riddle created by the changing kinetic behavior of glutathione reductase when, for instance, 2,4,6-trinitro-benzene sulfonate is used to study in vitro kinetics or when specific site directed mutagenesis is performed.

1. Introduction

This is a theoretical study, dedicated to Professor B. Pullman, addressing the study of the glutathione reductase catalytic mechanism. Professor Pullman has been a pioneer in the application of quantum mechanical methods to the study of organic and biologically important molecules [1]. Quantum biochemistry as autonomous research field arose from his early striving and we hope this work will illustrate one of its contemporary aspects.

Transition structures in vacuum appear to play a singular role in the description of enzyme catalyzed reactions [2–14]. As it is well acknowledged, the concept of transition state plays a central role in describing mechanistic aspects of chemical reactions and in the theory of rate processes [15–25]. However, although closely related to the idea of transition state, the use of a transition structure does not reduce to the tenets of the transition state theory [14]. Mathematically, this stationary point of the adiabatic electronic energy hypersurface corresponds to a saddle point of index one [26–28] where the relative signs for the amplitudes of the eigenvector associated with the unique negative eigenvalue (the transition vector

* Corresponding author.

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[26]) describes (at zero order) the interconversion step for a given mechanism. Thus, if reactants are molded by the enzyme into a geometry ressembling the structure of the saddle point of index one then the corresponding mechanistic channel can be opened [9–11]. Whether the pathway is used or not would depend on energetics (activation barrier) and dynamical fluctuation properties of the enzyme-substrate complex. The hypothesis of the present work is that for an enzyme catalyzed reaction showing different mechanisms, there exist different accessible transition structures.

Here, a transition structure (TS), or saddle point of index one, is theoretically characterized for hydride transfer between molecular fragments modeling the flavine and nicotinamide rings as they are found in glutathione reductase (GR). With this TS and related information, we discuss the apparent change of GR mechanism which is experimentally detected, for instance, in enzymes submitted to specific mutations [29], or when 2,4,6-trinitro-benzene sulfonate (TNBS) is used as substrate [30]; it is worth noticing that the reduced glutathione-linked $NADP^+$ reduction, catalyzed by yeast GR, follows a

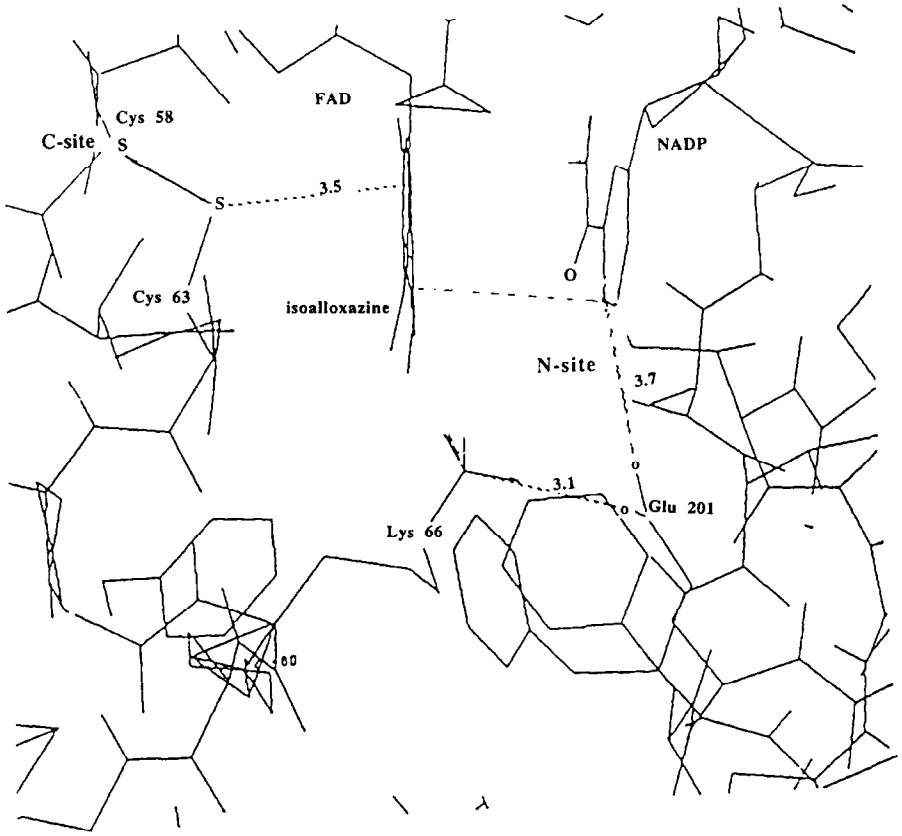


Fig. 1. Schematic view of the N-site in glutathione reductase. The plane of the isoalloxazine ring is almost perpendicular to the paper plane. The model was built as described in Ref. [37]. The distances are in Ångströms. The substrate binding site is not described; its tentative position is indicated as C-site. The distance between the C4 (nicotinamide ring) and N5 (FAD) is about 3.6Å. Note the presence of Lys66 just between both rings and the position of Glu201 at H-bond distance of Lys66. The redox active disulphide bridge is located behind the isoalloxazine ring when viewed from the nicotinamide ring. Note the lack of covalent interactions between FAD and the isoalloxazine binding site. The Tyr177 (*E. coli*) or Tyr 197 (human) referred to in the text (not shown in the figure) is to be found in a neighborhood of the nicotinamide ribose. Its location with respect to the nicotinamide ring is at the side opposite to the face interacting with the isoalloxazine ring.

sequential mechanism at high coenzyme concentration and a ping-pong mechanism at low $NADP^+$ concentration [31].

Early experimental work by Mannervik on GR catalyzed reduction of the TNBS with nicotinamide adenine dinucleotide phosphate (NADPH) showed that the kinetics in vitro should be described with a branched mechanism [30,32–34]. One kinetic loop proceeding via a ping-pong mechanism branched with a second loop corresponding to a sequential ordered kinetics. For this particular case, an ordered mechanism would mean first the formation of an enzyme–NADPH complex, followed by subsequent production of the ternary complex; substrate–enzyme–NADPH. A ping-pong mechanism would correspond to the binding of NADPH with the enzyme first, followed then by reduction of the intrinsic redox system of the enzyme and liberation of the oxidized coenzyme (this is the ping). The pong step corresponds to the formation of a reactive complex between the reduced enzyme and the substrate followed by liberation of the reduced substrate. These loops are coupled to each other via a common step, namely, the coenzyme NADPH binding. Mannervik's work was forgotten for a long time, presumably on the assumption that such peculiar mechanism was due to the particular substrate used an thereby had very little relevance under physiological conditions. In fact, for the natural substrate (oxidized glutathione, GSSG), the mechanism shows a ping-pong pattern [35,36] where, in the first step, NADPH leaves two electrons and one proton forming a reduced enzyme, symbolically designated as E-$FADH_2$. It is this species which subsequently binds GSSG and reduces it to two GSH molecules. The substrate (glutathione) binding site (G-site) is located in a region differing from the coenzyme binding site (N-site). Both sites are mediated by the flavine ring and the active disulphide bridge (cf. Fig. 1). Berry and coworkers showed that the mutated enzyme of *E. coli* at Tyr177 corresponding to Tyr197 of the human enzyme, changes the mechanism from ping-pong to sequential ordered with loss of activity when this tyrosine is replaced either by serine or glycine [29]. These results have not found a theoretical explanation.

Under physiological conditions the redox mechanism is described as an electron transfer mechanism [35,36]. Whether the actual pathway is achieved by one-electron steps or a two electron transfer [37], there seems to be no doubt that a hydride-like mechanism, such as the one found for liver alcohol dehydrogenase [38–40], is not operational here. Now, when the enzyme is mutated to Tyr177Gly to explain the change in mechanism, an attractive hypothesis would be to assume that during the redox process a *transposed hydride-like* transition structure is involved as a surrogate pathway. In order to theoretically support such an hypothesis we have searched after a hydride transfer transition structure for a model molecular system having the most relevant electronic elements. In this paper we report on a saddle point of index one for a model made by a nicotinamide and isoalloxazine ring structures. The calculations have been carried out at the Hartree–Fock level of theory with a 3-21G basis set for the atomic orbitals in the selfconsistent molecular orbital framework.

2. Method and models

The saddle point is located with the help of a local partitioning of the Hessian matrix (**H**) in two subspaces projected on internal geometric variables [41]. This procedure can not be general since the usual bond length, bond angle and dihedral angles cannot form a coordinate system for the entire configuration space [27,28]. This procedure is a local construct and the use of the term subspace is referred to a neighborhood of the corresponding stationary point. Thus, for a saddle point of index 1 (SPi-1) – which is usually referred to as first order saddle point in the chemical literature [27,28] – the variables having non-zero amplitudes in the eigenvector associated to the unique negative eigenvalue define the control space; this is symbolized by $\mathbf{H}_{cc}$. The other one is the complementary space $\mathbf{H}_{mm}$.

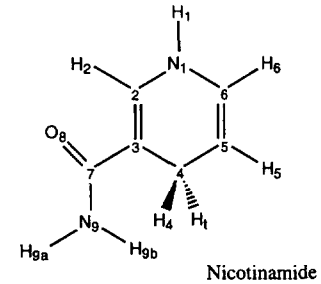
$$\mathbf{H} = \begin{vmatrix} \mathbf{H}_{cc} & \mathbf{H}_{cm} \\ \mathbf{H}_{mc} & \mathbf{H}_{mm} \end{vmatrix}.$$

The matrix elements of $\mathbf{H}_{cm}$ and $\mathbf{H}_{mc}$ are the cross-derivatives of the potential energy U with respect to coordinates belonging to the c- and m-subspaces. A meaningful partitioning implies that the matrix ele-

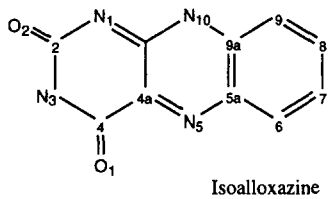
ments $\mathbf{H}_{cm}$ must be negligible with respect to the intra-block off-diagonal matrix elements in $\mathbf{H}_{cc}$. In practical cases, this is numerically tested by searching for the minimum number of internal coordinates yielding a unique negative eigenvalue and the transition vector amplitudes that in zero order are related to the chemical interconversion [41]. The control space usually contains a subset of minimal geometric variables that are sufficient for a proper description of the chemical interconversion process: the negative eigenvalue is sustained by this reduced set of coordinates. The minimal number of variables defines a reactive (active) space.

The partitioning and identification of a minimal set of coordinates defining the control space can not be seen as a numerical trick only. It reflects a relative independence of the internal degrees of freedom involved in the chemistry with respect to other subsidiary fragments. The subset of atoms included in the reactive space can be seen as a molecular moiety with dangling bonds. For particular cases, these latter are (covalently) saturated with atoms and/or molecular groups whose degrees of freedom are not strongly coupled with those of the active space. In the present case, the donor (C_4 nicotinamide) and acceptor (N_5 isoalloxazine) atoms together with the hydrogen exchanged between both form the reactive space (for numbering see Scheme 1). The orientation of the rings leading either to an endo or to an exo conformation and the atoms directly binded to the reactive space usually form the control space.

Löwdin's partitioning techniques [42] can be used to estimate the effect of $\mathbf{H}_{mm}$ on the reactive space. The coupling of the subspace associated to the negative eigenvalue (of the pre-diagonalized $\mathbf{H}_{cc}$ matrix) with the complementary space leads to an effective operator; the term added to the diagonal element is quadratic in the off-diagonal matrix elements and inversely proportional to the energy gap between the states connected by this matrix element and the bottom level. Since all the diagonal elements are numerically above (by definition) the unique negative eigenvalue, the gap is large and the perturbation is likely to be small. Therefore, if such a partitioning holds in vacuum, the TS geometry and fluctuation pattern are expected to be robust features to non interfering groups added to the m-space. It is not



Nicotinamide



Isoalloxazine

Scheme 1.

difficult to see that if one enlarges the m-subspace to encompass the surrounding protein, the active subspace may well be invariant to interactions with this enlarged system. Actually, surface complementarity would help to minimize interactions. It is probably for these reasons that a calculation in vacuum of transition structures may have relevance to discuss enzyme catalyzed reactions [9–11,14].

The definition of reactants and products requires some qualification. Active precursor and successor complexes are introduced to described the chemical inter-conversion step [14]. The structures of such complexes are determined by the geometry of the corresponding saddle point of index 1 in the sense that they have to be molded (deformed in several occasions) into a geometric arrangement as near as possible the geometry obtained for the SPi-1. By construction, such complexes are the reactive species opening a path towards the TS of the interconversion mechanism.

The results obtained for a number of enzyme catalyzed reactions [11] show that only the geometric arrangement of the SPi-1 in vacuum can be docked at the active site of the corresponding enzyme without bumping with the molecular walls. A complementarity in shape between the in vacuum structure and the active site has been found for all examples studied so far. Interestingly, the products and reac-

tants complexes obtained as minimum energy structures along an intrinsic reaction coordinate usually have intermolecular distances and/or orientations unsuitable to be docked at the active sites. To dock substrates at the active site, important molecular deformation must be introduced to them so that surface complementarity to the active site surface is achieved. The suggestion has been made [9,10] that the phenomenon of catalysis can be highlighted from the perspective of the reactants in vacuum as a specific binding process where the enzyme and substrates are molded into a geometry where the binded substrates ressemble as much as possible the one characterizing the SPi-1 in vacuum [9–11]. The extrapolation of in vacuum ab initio MO studies of first order saddle points to discuss enzyme catalyzed reactions raises important questions that have been addressed elsewhere [6,9–11,14].

Now, if there is a saddle point of index one for the model system representing GR, the enzyme-substrate system ought to have an alternative mechanism to accomplish the redox reaction. This is the hypothesis on which the present study rests.

Whether or not the system may take a particular route would depend upon structural and dynamical properties of the enzyme. This issue will be examined in the discussion section.

The elements of the molecular model used in the calculations are: (i) the flavine, represented by an isoalloxazine tricyclic ring; and (ii) the coenzyme NADP, represented by the N-protonated nicotinamide ring reduced at C_4. Atom numbering is depicted in Scheme 1. The hydride transfer mechanism is tested via a theoretical characterization of its corresponding SPi-1. Geometric parameters of the SPi-1 and a reactant complex identified along an intrinsic reaction coordinate calculation are consigned in Table 1. The fragments of the product supermolecule formed by the nicotinamide ring and the isoalloxazine ring appear oriented as the basic constituents belonging to the catalytic system.

The calculations have been performed with the GAUSSIAN 92 program [43]. Ab initio SCF MO calculations at a 3-21G basis set level [44] have been carried out. The Berny analytical gradient optimization routines [20,45] were used for the optimization. The requested convergence on the density matrix was 10^{-9} atomic units and the threshold value of maximum displacement was 0.0018 Å and that of maximum force was 0.00045 hartree/bohr. The na-

Table 1
Interatomic distances obtained with the geometry optimized fragments and the saddle point of index one obtained with a 3-21G basis set

Isoalloxazine			Nicotinamide		
bond	SPi-1	oxidized	bond	SPi-1	reduced
N5–C4a	1.3571	1.2716	N1–C2	1.3477	1.3662
C4a–C10a	1.4133	1.4599	C2–C3	1.3560	1.3307
C10a–N1	1.2920	1.2740	C3–C4	1.4406	1.5216
N5–C5a	1.4135	1.3892	C4–C5	1.4530	1.5204
C5a–C9a	1.3940	1.3942	C5–C6	1.3365	1.3187
C9a–N10	1.3800	1.3761	C6–N1	1.3831	1.3975
N1–C2	1.3776	1.3884	C3–C7	1.4933	1.4746
C2–N3	1.4083	1.4050	C7–O8	1.2223	1.2274
N3–C4	1.3715	1.3723	C7–N9	1.3530	1.3572
C4–C4a	1.4340	1.4843	C4–H4	1.0768	1.0895
C5a–C6	1.3871	1.3925	C4–Ht	1.4071	1.0895
Isoalloxazine ring					
C6–C7	1.3763	1.3706	C7–C8	1.3915	1.3974
C8–C9	1.3766	1.3736	C9–C9a	1.3918	1.3915
C2–O2	1.2102	1.2036	C4–O1	1.2311	1.2075

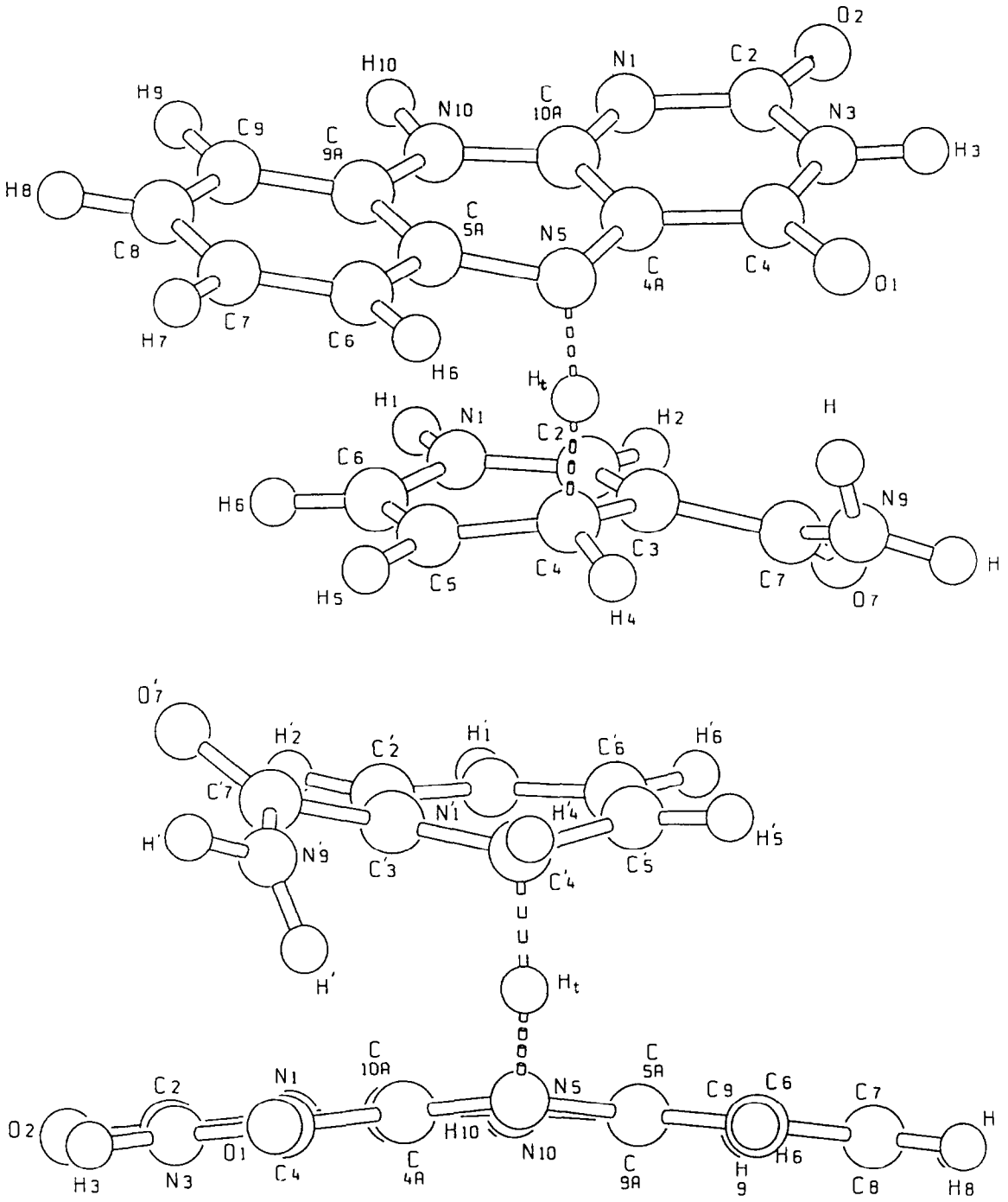


Fig. 2. Two views of the geometry corresponding to the saddle point of index one. The atom numbering is given in Scheme 1. In the upper part, isoalloxazine (above) and nicotinamide ring (below) are depicted in their endo configuration and the transferred hydrogen is singled out (H_t). In the lower part, nicotinamide ring is displayed above the isoalloxazine ring in a view where the butterfly conformation of isoalloxazine can be seen.

ture of the stationary point was established by calculating and diagonalizing the Hessian matrix. Correlation effects in this type of reaction are energetically important [40] but they do not affect too much the geometric issues discussed here [46].

3. Results

3.1. Saddle point of index 1

The geometry of the saddle point of index 1 for the hydride transfer from nicotinamide and the isoalloxazine ring is presented in Fig. 2.

The saddle point of index one presents several interesting features. (1) The structure is syn with the angle between C4HtN of 158° which is 18° larger than the one found for the SPI-1 for the hydride transfer to pyridinium cation from methanolate [2]. (2) The forming N5–Ht is shorter than the breaking bond C4–Ht. (3) The nicotinamide ring is slightly deformed while the isoalloxazine ring shows a butterfly deformation (angle $\approx 170°$). (4) The charge transfer to the isoalloxazine ring is ≈ 0.56 au which is slightly larger than the one transferred to the pyridinium cation; the net atomic charge at Ht is positive (≈ 0.34). (5) There is a strong coupling between the transferred hydrogen and the dihedral (out of plane) of the hydrogen staying at C4. (6) All diagonal force constants in the selected frame of internal coordinates are positive. (7) The negative eigenvalue is the result of a number of coupling of internal degrees of freedom.

Due to the absence of covalent interactions between the isoalloxazine ring in FAD with the enzyme, the butterfly deformation can be attained without working against strong forces.

3.2. Product and reactant complexes

Descents from the SPi-1 along the intrinsic reaction coordinate (IRC) [47,48] permit characterizing a reactant and a product complexes. For the geometry optimized minimum energy structures, the intermolecular distances are far too long to be adapted without straining at the active site. However, along the IRC, there are hydrogenated isoalloxazine product complexes whose intermolecular distances and spatial orientation with respect to the oxidized nicotinamide moiety in vacuum are compatible with the spatial restriction set up by the active site. This constrained model can be overlapped with a molecular arrangement as it is found the protein and shown in Fig. 1 [37].

Note that the relative orientation in the reactant complex (not shown) corresponds to the model reported by Sustman and Schulz [49], as well as by Horjales et al. [37]. The distance between the two rings can change from about 3.2 to 3.4 Å. The isoalloxazine ring is planar.

The constrained product complex has an interesting feature: the isoalloxazine ring is bent with a butterfly angle of 169°, the hydrogen bounded to N5 is therefore oriented towards the N-site. A typical hydride donor acceptor distance corresponds to about 3.2 Å. Note that this product complex would only be an intermediate in the ordered mechanism as it is discussed below and the butterfly conformation forces the hydrogen to rest at the N-site.

4. Discussion

Let us consider the ordered sequential loop of the branched mechanism. In Eq. (1) below it is indicated the equilibrium between the reduced coenzyme and free enzyme (EFAD) and the productive binding complex [E′FAD–NADPH]. The enzyme's conformational changes that take place after coenzyme binding are symbolized with E′. In particular, at the N-site in the apoenzyme Tyr 197 (177 in *E. coli*) is closing this site from the solvent. This residue makes a large movement on NADPH binding [35]. In Eq. (2) it is represented the transposed hydride transfer step. The substrate GSSG is assumed to have a higher affinity with the reduced E′FADH than with the oxidized form E′FAD. In the equation quoted below: FAD = flavine; E-enzyme; E′HFAD = enzyme protonated at Lys66 and Glu201 (human); $E'HFAD^-$ enzyme as before but reduced FAD (negatively charged) with charge delocalization over the redox active S–S bridge (cf. Fig. 1); $NADP^+$ oxidized coenzyme.

$$EFAD + NADPH \rightarrow [E'FAD\text{–}NADPH], \quad (1)$$

$$[E'FAD\text{–}NADPH] \rightarrow [E'FAD\text{–}H\text{–}NADP], \quad (2)$$

$$[E'FAD\text{–}H\text{–}NADP] + GSSG$$

$$\rightarrow \text{GSSG–[E'FAD–H–NADP]}, \quad (3)$$

$$\text{GSSG–[E'FAD–NADP]} \rightarrow \text{GSSG–[E'HFAD–NADP}^+\text{]}, \quad (4)$$

$$\text{GSSG–[E'HFAD}^-\text{–NADP}^+\text{]} \rightarrow \cdots \rightarrow 2\text{GSH} + \text{NADP} + \text{EFAD}. \quad (5)$$

The reactant complex [E'FAD–NADPH] would correspond to Pai and Schulz guess with a geometry near the one calculated in this work. The important is the existence of the saddle point of index 1 which would prompt the interconversion step (2) *in absence* of the substrate. Remember that the transition zone is found at ring-ring distance of 2.59 Å while in the X-ray structures this distance fluctuates around 3.1–3.6 Å [37]. Thus, one way to form the product complex [E'FAD–H–NADP] would be to force the protein into a fluctuation pattern in which the probability is enhanced to attain distances between the donor-acceptor centers compatible with those required by the SPi-1. If such fluctuations are allowed for, the probability of forming the product complex would increase. Now, the existence of a product complex obtained following the IRC opens the possibility to form a temporarily FAD-reduced [E'FADH–NADP]. Here, the transferred hydrogen is sticking towards the N-site. This result agrees with the consensus view that no direct hydrogen transfer exists during catalysis between the N-site and the G-site. Furthermore, the electrons are then concentrated in the bonding region of the N5–H. One would then expect that the substrate binds to the G-site to form a complex of the type (3) without reaction yet.

Now, if the hydride transfer mechanism is to agree with common wisdom, a deprotonation step at the N-site via Lys66 after substrate binding appears to be a reasonable assumption. In Eq. (4), GSSG–[E'HFAD–NADP] corresponds to a deprotonated flavine with the proton now sitting at Lys66. This process would make necessary the presence of a proton acceptor group for the otherwise protonated lysine. This proton receptor role should be played by Glu201 [37], which is conserved residue. The formation of GSSG–[E'HFAD$^-$–NADP$^+$] would open a channel for the reduction of GSSG as the electrons of NADPH are left behind on the flavine–disulphite system (E'HFAD$^-$). The actual electronic mechanism herein proposed appears as a *transposed hydride transfer* as the protons required to proceed along the reaction path are given at the G-site by another proton shuttle structure and the solvent accessible to this site [35]. The isoalloxazine ring here does transfer electrons but not a proton to the substrate. The proton relay systems found at the N- and G-sites increase the efficiency of the catalytic mechanism [11].

The difference in mechanism detected between the mutant Tyr177Gly (*E. coli*) and the wild type can be understood in terms of a change in the fluctuation pattern of the ribose and nicotinamide ring. Tyr177 is just behind this ring at the N-site interacting with the ribose ring. For the wild type, its presence would likely ensure interactions holding NADP ring at distances where the SPi-1 for hydride transfer cannot be attained. Mutation of this residue by glycine or serine would reduce the interactions with the ribose thereby increasing the probability to attaining the ring-ring distances where SPi-1 region is to be found. Formation of the product complex blocking the electron transfer pathway.

As there is no actual molecular dynamics simulations on such systems, the ideas discussed so far can be considered as plausibility arguments. Still, they are sufficient to understand that GR may have two possible mechanistic pathways. The fact that a strong electron attractor substrate such as TNBS has a branched mechanism may also be understood in terms of the same set of ideas. The riddle set up by Mannervik's kinetic study can be understood now in terms of the existence of two alternative mechanisms to accomplish the redox equivalent transfer.

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