The role of tetrahydrobiopterin in catalysis by nitric oxide synthase

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Electronic structure calculations show that the cofactor H_4B can be a key factor in a proton transfer relay in nitric oxide synthase, and that 4-amino-H_4B cannot fulfill this role.

Nitric oxide (NO) is a signalling molecule that participates in a wide range of physiological and pathophysiological processes in mammals.[1] The biosynthesis of NO is carried out by a family of enzymes, nitric oxide synthases (NOS, EC1.14.13.39) that catalyse the conversion of L-arginine to citrulline and NO in two consecutive hydroxylation reactions with N^G-hydroxy-L-arginine (NHA) as the intermediate.[2] NOS, unlike other P450-type enzymes, requires tetrahydrobiopterin (H_4B) as a cofactor.[3] Other tetrahydropteridines are competent cofactors, but a notable exception is 4-amino-tetrahydrobiopterin (4-amino-H_4B), which is a strong inhibitor of NOS catalysis.[4] One function of H_4B, that of a one-electron donor to the heme–oxygen complex, Fe(II)O_2, is now generally accepted.[4] However, the inhibitor 4-amino-H_4B has similar electrochemical properties to those of H_4B,[5,6] suggesting a function of H_4B in addition to reduction. Crystallographic studies have revealed essentially identical binding modes, hydrogen bonding interactions and surrounding water structure for both the active H_4B and inactive 4-amino-H_4B, indicating that structural differences cannot explain the difference in their activities.[7] These X-ray studies have also suggested a possible proton-coupled electron transfer mechanism involving H_4B. A possible pathway for this transfer from H_4B to the heme-bound ligand involving the propionate side chain on the heme porphyrin, has been suggested and is shown in Fig. 1.[8] Thus, there is the strong suggestion that H_4B might be a key component of a proton relay pathway in NOS, essential for the proposed protonation of the Fe(II)O_2^- complex. The ultimate goal is to model all of the steps of this complex reaction including the proton-coupled electron transfer step, which is a particularly formidable challenge. However, much insight can be gained by studying the individual steps at the important centres and seeing how these are affected by changes in chemical structure. Cho and Gauld[9] have modelled the initial complexes and intermediates, without the explicit inclusion of the pterin cofactor. We are here concerned with another important aspect of the overall reaction, that of the role of the pterin cofactor.

However, there are a number of possible protonation motifs of the bound pterin, the heme carboxylate and the neighbouring residues, particularly arg375, which is hydrogen-bonded to the carbonyl oxygen atom of the pterin. The identification of the preferred structure, before and after the loss of an electron from the pterin, is central to understanding a possible proton transfer mechanism. This information is difficult to obtain experimentally, in view of the quite small differences expected in the bond lengths between heavy atoms in the different structures. Computations can play an important role in predicting the relative energetics of these structures, and such a study is described here.

We have used electronic structure calculations to find the most stable structures of the pterin–heme carboxylate pair, before and after an electron has been ionised from the pterin. We have studied a cluster which contains the pterin, nearby residues, a number of crystallographic water molecules, and HCO_2^- to model the heme carboxylate. The important residue arg375, which hydrogen bonds to the oxygen or nitrogen in H_4B and 4-amino-H_4B respectively, was modelled as $H_2NC(=N^+H_2)NH_2$ (Fig. 2).[10] The cluster also included trp457, modelled as CH_2O. The heme centre is $\sim$15 Å from the pterin so that it is unlikely to influence the electronic structure of the pterin region.

The calculations were carried out at the density functional theory (DFT)[11] level, employing the B3LYP functional. Geometry optimization of the various structures was carried out using the 6-31G* basis, and subsequent energies were evaluated using the larger 6-311+G** basis (B3LYP/6-311+G**//B3LYP/6-31G*),[12] which we label model I. During the optimisation procedure the positions of the carboxylate carbon and arg375 α-nitrogen atom were fixed at the crystallographic positions. The electrostatic effect of the bulk enzyme was included using the polarizable continuum model (PCM)[13] with a dielectric constant of 4 (B3LYP/6-311+G**(PCM)//B3LYP/6-31G*), which we label model II. We first consider the neutral and ionised states of H_4B.

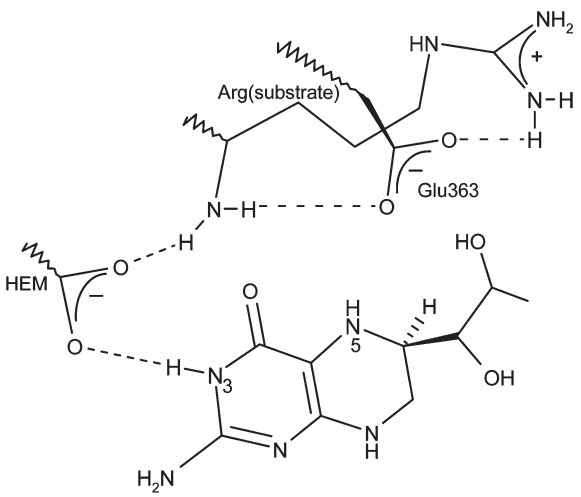

Fig. 1 Proton and electron transfer pathway from pterin to L-arginine *via* heme carboxylate and glu363.[8]

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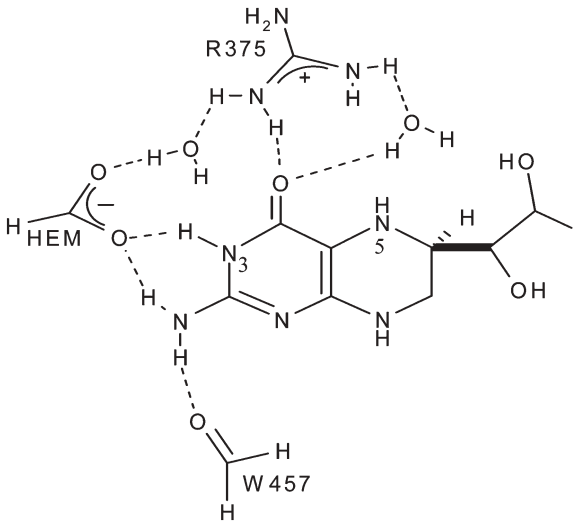

Fig. 2 Tetrahydropterin cluster model.

We find that for the isolated pterin, the tautomeric enol form (**C**), which is formally aromatic, is the most stable, as is generally found for similar heterocycles, being 22.2 kJ mol^{-1} lower in energy than the carbonyl form (**A**). In the cluster environment there is preferential stabilisation of the carbonyl form (**A**), due in large measure to interaction with the positively charged arginine residue, leading to a slight preference for **A** compared to the enol form (**C**) (Table 1), which is enhanced in the continuum model. We also see (Fig. 3) that the structure of the enol form now involves a neutral arg375 residue and a protonated heme carboxylate. Thus, although the pterin remains neutral in this structure, proton transfer involving arg375 and the carboxylate has taken place. In addition, we find a third structure (**B**), in which the N3 proton of the carbonyl form has transferred to the carboxylate oxygen, but here arg375 is still positively charged, leaving a negatively charged aromatic pterin. This structure and the carbonyl form are not only preferentially stabilised compared to the enol form by arg375, but also by the continuum, since there is more charge separation in **A** and **B** than in the enol form (**C**). Thus, before electron transfer has occurred, both structures **A** and **B** could well be present, although our calculation slightly favours the carbonyl form **A**. The relative energies of these three structures are significantly altered upon electron ionisation from the pterin (Table 1). The lowest energy structure now clearly corresponds to the ionised enolate **B**, in which proton transfer from N3 to the carboxylate has taken place to give an effectively neutral pterin. This structure is at a considerably lower energy than either of the two alternatives

Table 1 The relative energies (kJ mol^{-1}) of H_4B and 4-amino-H_4B in the active site model

		Neutral		Cation	
		Model I	Model II	Model I	Model II
H_4B	**A**	0.0	0.0	38.1	28.7
	B	1.5	5.0	0.0	0.0
	C	2.6	15.8	35.9	47.1
4-amino-H_4B	**D**	8.2	1.6	0.0	0.0
	E	17.0	15.5	55.7	41.2
	F	0.0	0.0	30.4	52.4

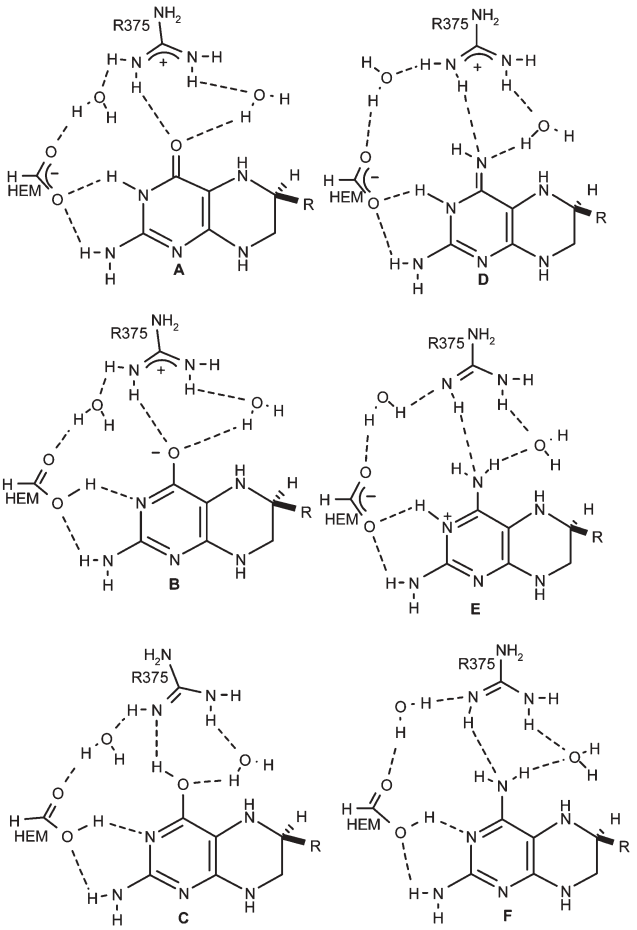

Fig. 3 The tautomeric structures of H_4B (**A**, **B** and **C**) and 4-amino-H_4B (**D**, **E** and **F**), R = (CHOH)(CHOH)(CH_3).

(ionised **A**, **C**), both with and without the continuum. These latter two structures correspond to ionisation of the neutral carbonyl and enol forms, which results in a positively charged pterin. Here the unfavourable Coulombic repulsion between the positively charged pterin and the protonated arg375 is presumably the source of their relatively high energies. Thus, we predict that the ionisation of the pterin results in a simultaneous proton transfer from N3 of structure **A**, to the heme carboxylate, in agreement with the suggestion of a coupled electron–proton transfer in the activation process. Our proposal that the H_3B radical corresponding to **B** results from electron loss from H_4B is in line with EPR studies,[14] which suggest there is significant spin density on N5 of the radical, our calculations predicting $\sim$0.20 electron spin density at N5 for radical (**B**) (models I and II).

Turning now to 4-amino-H_4B, we find that, both for the isolated pterin and for the cluster model in the absence of the continuum, the di-amino form (**F**), is, as expected, strongly preferred over the imine tautomer (**D**), or in the case of the cluster, the protonated amino structure (**E**). In the cluster, the interactions involving structure **F** are similar to those of the enol form (**C**) of H_4B, in which arg375 is in the imine form, and the heme carboxylate is protonated. In the presence of the continuum, (model II), structure **D**, which involves the imine form of the pterin, and the charged forms of arg375 and the heme carboxylate, is preferentially stabilised and is now close in energy to structure **F**.

Thus, as we found for H_4B itself, before electron transfer there could well be a mixture of tautomers present. However, upon ionisation of 4-amino-H_4B, the most stable structure clearly corresponds to the ionised imine form **D**, in which the highly basic guanidine group has been protonated. Ionization of structure **F** of 4-amino-H_4B increases the acidity of the hydrogens of the amino group at C4, leading to proton transfer to the arginine group. At the same time N3 is rendered more basic and accepts the heme carboxylate proton leading to structure **D**. This is in stark contrast to the case of H_4B itself, where the heme carboxylate is definitely protonated after electron transfer.

Our calculations have shown that the major difference between the two cofactors is that the proposed mechanism involving both electron and proton transfer to the enzyme–substrate structure can occur for the active H_4B cofactor, whilst for the inactive 4-amino-H_4B, this mechanism cannot take place, since the N3 of the pterin rather than the carboxylate is protonated after electron ionisation. In line with electrochemical studies, our calculations also predict very similar ionisation energies for H_4B and 4-amino-H_4B, the values being 533.8 and 543.4 kJ mol^{-1} respectively (model II), so that the different behaviour of the two pterins cannot be due to differences in the ionisation energies.

Our calculations thus support the suggestion that, in addition to acting as an electron donor, H_4B also participates in a proton transfer relay to the substrate, and that the reason for inhibition by 4-amino-H_4B is associated with its inability to participate in such a relay mechanism. This computational approach might also be useful in helping to design new cofactors.

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