A Vibrational Structure of 7,8-Dihydrobiopterin Bound to Dihydroneopterin Aldolase†

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ABSTRACT

Dihydroneopterin aldolase (DHNA) catalyzes the conversion of 7,8-dihydroneopterin to 6-
hydroxymethyl-7,8-dihydropterin and glycolaldehyde. An inhibitor of the enzyme, 7,8-
dihydrobiopterin, free in solution and bound in its complex with the enzyme has been studied by
Raman difference spectroscopy. Using isotopically labeled 7,8-dihydrobiopterin and normal
mode analyses based on \textit{ab initio} quantum mechanic methods, we have positively identified
some of the Raman bands in the enzyme bound inhibitor, particularly the important N5=C6
stretch mode. The spectrum of the enzyme bound inhibitor shows that the pKa of N5 is not
significantly increased in the complex. This result suggests that N5 of 7,8-dihydroneopterin is
not protonated before the bond cleavage of 7,8-dihydroneopterin during the DHNA catalyzed
reaction as has been suggested. Our results also show that the N5=C6 stretch mode of 7,8-
dihydrobiopterin shifts 19 cm\textsuperscript{-1} upon binding to DHNA. Various possibilities on how the
enzyme can bring about such large frequency change of the N5=C6 stretch mode are discussed.
INTRODUCTION

Dihydroneopterin aldolase (DHNA) catalyzes the conversion of 7,8-dihydroneopterin to 6-hydorxymethyl-7,8-dihydropterin and glycolaldehyde (Scheme I). This enzyme is an important link in the pathway of folate biosynthesis in most microbes. It and the other enzyme important to folate pathway, dihydrofolate reductase (DHFR), are important potential drug targets for antibacterial chemotherapy. Recently, the X-ray crystal structures of 7,8-dihydroneopterin aldolase from Staphylococcus aureus and its complex with 6-hydorxymethyl-7,8-dihydropterin were determined (1). There are several key residues at the active site that interact directly with bound substrate. These are Glu74, Lys100, Glu32, a structural water molecule close to N5 of the pterin ring, Val52, and several mainchain amino groups as shown in Scheme I. All these factors can result in substantial distortions of the ground state electronic properties of bound substrates; some are important to binding while others are important to catalytic turnover.

The vibrational spectrum of a molecule reports on the bonds orders of molecular bonds and is very sensitive to the small changes in the distribution of electrons within bonds that come about when an enzyme binds its substrate. The changes are a fingerprint of the enzyme-substrate interactions that exist in the ground state. Raman difference spectroscopy is well suited for the measurement of the Raman spectrum of a ligand bound to an enzyme (2). In this study, we have applied the technique to the DHNA/7,8-dihydrobiopterin (Scheme II) enzyme/substrate complex. Previous studies have shown that 7,8-dihydrobiopterin is an inhibitor to the DHNA catalyzed conversion of 7,8-dihydroneopterin, and it also promotes the formation of enzymic tetramer, an inactive form of the enzyme, from the active octamer (Unpublished results). In addition, our current studies show that 7,8-dihydrobiopterin is a poor substrate of DHNA. It is converted to an as yet to be fully characterized species, but at a reaction rate slow enough so that Raman difference spectroscopy is feasible. Since 7,8-dihydrobiopterin and 7,8-dihydroneopterin (the
normal substrate of dihydroleptine aldolase) are very similar structurally, particularly with regards to the pterin ring and close to the bond breaking point in the catalyzed reaction and also undergoes enzymatic catalysis, we use bound 7,8-dihydrobiopterin as a mimic for the Michaelis complex of this enzyme.

The binding site structure of DHNA around the pterin ring of the substrate is very much like that of dihydrofolate reductase (3-5). For example, the N2-C2-N3 moiety of the dihydropterin ring forms hydrogen bonds to Glu74, similar to those found for the structurally conserved Asp in dihydrofolate reductases (DHFR). This, other structural similarities, and chemical considerations, prompted the suggestion that the reaction mechanism of DHNA may be similar in some respects to DHFR (1). In particular, it has been postulated that the electron flow out of the C-C bond that is broken by DHNA (scheme I) could be stabilized by protonation of N5 of the pterin ring. A recent study using Raman difference spectroscopy of the electronic structure of substrates bound to E. coli dihydrofolate reductase showed that the pKa of N5 is raised from 2.6 in solution to 6.5. This can be determined in titration studies using vibrational spectroscopy since the N5=C6 stretch 'marker' band is located near 1650 cm⁻¹ or 1675 cm⁻¹ for unprotonated or protonated N5, respectively, exhibiting then easily measured and well separated bands (6). Hence, a particular focus of this investigation is the protonation state of N5 in the pterin ring for substrate bound to DHNA. We found little, or no, protonation of N5 when 7,8-dihydrobiopterin binds to DHNA. However, other unusual binding induced changes to the substrate's electronic structure within the pterin ring are observed.

MATERIALS AND METHODS
7,8-Dihydroneopterin, 7,8-Dihydrobiopterin and [5-15N] 7,8-Dihydrobiopterin were obtained from the laboratory of Dr. B. Schircks (Jona, Switzerland). Dihydroneopterin aldolase (DHNA) was prepared according to the published procedures (1). The sample was then stored at -80°C in 20 mM tris at pH 7.5, 100 mM NaCl, 5% glycerol. The sample for spectroscopic measurements was prepared by extensive dialysis of the DHNA sample in 25 mM tris at pH 7.8, 150 mM NaCl buffer, followed by concentration with a Centricon30 centrifuge concentrator (Amicon, Lexington MA) to the desired concentration. The concentrations of DHNA and 7,8-dihydrobiopterin were determined spectroscopically, using a molar extinction coefficients of 83000 M⁻¹ cm⁻¹ at 280 nm for the DHNA octomer and 6170 M⁻¹ cm⁻¹ at 330 nm (7) for 7,8-dihydrobiopterin. The binary complex of DHNA/7,8-dihydrobiopterin was prepared by mixing DHNA and 7,8-dihydrobiopterin in a molar ratio of 1:1. Typical concentration of the DHNA/7,8-dihydrobiopterin complex for UV-Vis absorption studies was approximately 0.05-0.5 mM (active site), and for Raman studies was about 4 mM.

Spectroscopy. The Raman spectra were measured using an optical multichannel analyzer (OMA) system. The OMA system uses a Triplemate spectrometer (Spex Industries, Metuchen, NJ) with a model DIDA-1000 reticon detector connected to an ST-100 detector (Princeton Instruments, Trenton, NJ). Details of the system can be found elsewhere (2). The 514.5 nm line from an argon ion laser (Model 165, Spectra Physics, Mountain View, CA) was used to irradiate the sample (~100 mW). Separate spectra for enzyme and enzyme•inhibitor complexes in solution, approximate concentration of 4 mM, were measured using a special split cell (the volume of each side being about 30 µl) and a sample holder with a linear translator as previously described (2). The spectrum from one side of the split cuvette is taken, the split cell is translated, and the spectrum from the other side is taken. This sequence is repeated until sufficient signal to
noise is obtained. A difference spectrum is generated by numerically subtracting the sum of the spectra obtained from each side. In general, the two summed spectra do not subtract to zero, as judged by the subtraction of well known protein marker bands (for example, the amide-I, amide-III, and the 1450 cm\(^{-1}\) bands, the latter band being especially useful since it is generally not affected by protein conformational changes). These protein marker bands are determined from their band widths (generally much broader than those from spectra of bound substrates) and their characteristic positions. Hence, one summed spectrum is scaled by a small numerical factor, generally between 1.05-0.95, which is adjusted until the protein bands are nulled (see e.g. 8). The same control procedures were performed on all the difference spectra results herein. Resolution of the spectrometer is 8 cm\(^{-1}\) for the present results. A spectral calibration is done for each measurement using the known Raman lines of toluene, and absolute band positions are accurate to within ±2 cm\(^{-1}\). None of the spectra presented here have been smoothed.

Calculations. The \textit{ab initio} calculations were carried out on the models by Hatree-Fock method with the 6-31g** basis set, as implemented in Guassian 98. The geometries of the model compound of 7,8-dihydrobiopterin were first optimized energetically and then the vibrational normal modes were calculated using the same basis set. True local minimum on the potential surface of the complexes for the geometry-optimized complexes were verified from the vibrational frequency calculations in which no imaginary frequency was found. In all cases, a stable structure of the model compound is achieved without any geometry constraint.

RESULTS

When 7,8-dihydrobiopterin binds to DHNA, it slowly undergoes product formation. Figure 1 shows the UV-Vis absorption measurements of the DHNA/7,8-dihydrobiopterin complex at 1,
10, 25, 55, 80, 120, 150, 180 and 360 min time intervals, respectively, after mixing. The sample pH was 7.8 and the temperature was set to 32°C. The initial spectrum has a $\lambda_{\text{max}}$ of 332 nm, slightly red shifted from the 328 nm $\lambda_{\text{max}}$ of 7,8-dihydrobiopterin in solution at the same pH. DHNA then slowly converts 7,8-dihydrobiopterin into a new species, which has an absorption band at 423 nm. Separate kinetic experiments of this complex at various pH values indicate that the reaction rate is lowered by about 2.5 fold for pH = 6.5. The intensity increase of the 423 nm absorption band can be fitted with a first order exponential function. Based on this result and the observation of a clear isosbestic point found in the absorption spectra of complex at various stages of the reaction (Figure 1), it is reasonable to conclude that only one product is formed. A similar spectral change occurs for 7,8-dihydroneopterin, the normal substrate of DHNA. However, no isosbestic point was found and the kinetics can not be fitted with a single exponential function. This product of the reaction of 7,8-dihydrobiopterin is not due to protonation of N5 of the pterin ring; the red-shift is much too large. Titration studies of 7,8-dihydrobiopterin in solution show that when N5 of 7,8-dihydrobiopterin is protonated, a new absorption band at ~365 nm appears. If protonated 7,8-dihydrobiopterin has a similar absorption in DHNA, we can estimate the percentage of this form in the DHNA/7,8-dihydrobiopterin complex from its absorption spectrum taken just after mixing. On this basis, less than 10% of N5 is of the protonated form in the enzyme/7,8-dihydrobiopterin complex. Furthermore, there is no detectable increase of this form when the sample pH is changed to 6.5.

Figure 2 shows the Raman spectra of 7,8-dihydrobiopterin in solution at pH 6.5. The pKa of N5 is 2.6 (6) so that N5 of pterin ring is unprotonated in the spectra of Figure 2. There are four major peaks above 1400 cm$^{-1}$ evident in panel a, at 1480, 1559, 1605 and 1636 cm$^{-1}$, respectively. The band at 1636 cm$^{-1}$ can be assigned to the C6=N5 stretch based on its 17 cm$^{-1}$
down shift in the $^{15}$N$_5$ labeled compound spectrum (Figure 2b; 6). The other three bands have not been assigned before. However, with the aid of \textit{ab initio} frequency calculations at the HF/6-31g** level performed on a model compound for 7,8-dihydrobiopterin, preliminary assignments of these bands can be made. Here we need to point out that the chemical bond lengths, especially the double bonds which contain oxygen or nitrogen, are underestimated by \textit{ab initio} methods at the Hatree-Fock level compared with those observed values. This is due to the neglect of electron correlation forces in the calculations, the limited basis set, and also that the calculations are 'gas phase' which do not treat the interactions with solvent. Consequently, the calculated stretch frequencies of these bonds are typically overestimated (15-20\%) compared to the observed values. Nevertheless, the overestimation of the calculated frequency differences compared to experimentally determined values tends to remain constant. Hence, by applying a uniform scaling to the calculated frequencies, reasonably accurate normal mode assignments can be made based on the calculations. Our previous experience on such calculations suggests that, in many cases, the calculations provide a reasonable description of the normal modes and their response to isotopic labeling or to environmental change around the molecule (9-11).

It is well known that the Raman bands in the spectral region between 1400 – 1700 cm$^{-1}$ are consist of normal modes mainly due to C=C, C=O, C=N stretch motions and CH bending motions. For 7,8-dihydrobiopterin, there are four double bonds and thus we expect to observe at least four Raman bands in these region, although some of the modes may contain more than one double bond stretch characters. According to the \textit{ab initio} frequency calculations on an isolated 7,8-dihydrobiopterin molecule, there are five vibrational modes that have relatively strong Raman intensities in this region. The calculations predict that the band with lowest frequency in this region, at 1480 cm$^{-1}$ in Figure 2a, which is not sensitive to the $^{15}$N$_5$ labeling but shifts
down by 6 cm\(^{-1}\) to 1474 cm\(^{-1}\) when the exchangeable protons of 7,8-dihydrobiopterin are deuterated as occurs for solution samples in D\(_2\)O (panels b and c of Figure 2, respectively), is likely due to the in-plane bending mode of the C\(_7\)H\(_2\) group of the dihydropterin ring. There are two ways to make assignments of the two bands at 1559 and 1605 cm\(^{-1}\). The first is to assign the 1559 cm\(^{-1}\) band to a C=C ring stretch which also contains contribution from N8H bend. This mode is not sensitive to the \(^{15}\)N\(_5\) labeling (Figure 2b) but shifts down 38 cm\(^{-1}\) for deuterated samples (Figure 2c) which removes coupling to NH bending motions because of the large downshift of a ND bend. In this case, the 1605 cm\(^{-1}\) band in Figure 2a is assigned to the C1=N2 stretch with contributions from N2H and N3H bending motions. In D\(_2\)O, the frequency of this mode would downshift to 1577 cm\(^{-1}\) as observed (Figure 2c) because its coupling with the NH bending is removed. A second way to assign these two modes that is compatible with the calculations is that the C1=N2 stretch and C=C stretch of the dihydropterin ring are coupled to form an in-phase and an out-of-phase pair. The in-phase mode has a higher Raman intensity and lower frequency, the 1559 cm\(^{-1}\) band in Figure 2a can be assigned to this mode. The out-of-phase mode has a lower Raman intensity and higher frequency, and the 1605 cm\(^{-1}\) band in Figure 2a can be assigned to this mode. Since both of these modes have contributions from the ring NH bending motions, significant frequency shifts are predicted for deuterated samples, which is consistent with experimental observations.

The \textit{ab initio} calculations also predict that the two highest frequency modes in this region are due to the C\(_6\)=N\(_5\) and C\(_4\)=O stretch modes. The C\(_6\)=N\(_5\) stretch mode is predicted to have the highest Raman intensity and its frequency is insensitive to NH dueteration (unless N\(_5\) is protonated, which is not the case here). Such predictions are in very good agreement with the
experimental observations (see Figure 2a, 2c). The calculations also predict that the frequency of the C4=O stretch mode, predicted to have significantly lower (0.2-0.1 times) Raman intensity than that of C6=N5 mode, is very sensitive to the strength of hydrogen bonding to the C4=O oxygen. In vacuum, its frequency is higher than the C6=N5 stretch but, if a hydrogen bonded water molecule is included in the calculations, its frequency is comparable to the C6=N5 stretch mode. This prediction is supported by the observation of the Raman and IR spectra of 7,8-dihydrobiopterin in DMSO (data not shown). The C6=N5 stretch shifts down by 6 only cm\(^{-1}\) for 7,8-dihydrobiopterin in DMSO compared to its value in water while a new Raman band is observed at 1658 cm\(^{-1}\) that is insensitive to \(^{15}\)N5 labeling (data not shown). Based on this and the fact that the 1658 cm\(^{-1}\) band is very strong in IR, the 1658 cm\(^{-1}\) band can be easily assigned to C=O stretch for 7,8-dihydrobiopterin in DMSO. Thus, it is reasonable to assume that the C4=O stretch mode of 7,8-dihydrobiopterin in water is hidden somewhere under the intense C6=N5 stretch mode at 1636 cm\(^{-1}\) in Figure 2a.

Our calculations also predict that, under certain conditions, the C4=O stretch and C6=N5 stretch can be mixed. In this case, the Raman intensity of the C4=O becomes significantly higher by borrowing intensity from the C6=N5 stretch. Such results provide a rationale for the observation of the doublet at 1623/1638 cm\(^{-1}\) in 7,8-dihydrobiopterin in D\(_2\)O (Figure 2c). Apparently, this doublet is due to the coincidental mixing of the two stretch modes so that the intensity of the C4=O mode becomes much stronger. This interpretation is further supported by the observation that only a single band is observed at 1614 cm\(^{-1}\) (data not shown) for the \(^{15}\)N5 labeled compound in D\(_2\)O. The band at 1623 cm\(^{-1}\) can be assigned to the C4=O stretch while
that at 1638 cm\(^{-1}\) to C6=N5 stretch since the former is strong in the IR spectrum compared to the latter (data not shown).

Since 7,8-dihydrobiopterin is slowly converted to product during the Raman measurements, the difference spectrum between DHNA/7,8-dihydrobiopterin complex and DHNA obtained in the usual way (see Methods) will contain Raman bands from the bound enzyme product of 7,8-dihydrobiopterin. Thus, an extra Raman spectrum of DHNA/7,8-dihydrobiopterin complex was taken after the reaction completed, as monitored by UV-Vis absorption. Since it has been shown that there is just one product (see above and Figure 1) and the product has characteristic Raman bands that are distinct from those of 7,8-dihydrobiopterin (data not shown), it is possible to remove all of the peaks of the enzyme product from the Raman difference spectrum. This was achieved by obtaining two difference spectra between DHNA/7,8-dihydrobiopterin complex and DHNA: the first from the freshly prepared DHNA/7,8-dihydrobiopterin and the second from the DHNA/7,8-dihydrobiopterin complex three hours later after the reaction was completed. An appropriate amount of the second difference spectrum is subtracted from the first to remove the Raman peaks due to the enzyme product.

The results of this procedure are presented in the difference spectra of Figure 3. In these spectra the major bands are from bound 7,8-dihydrobiopterin, and the minor bands are either from unbound 7,8-dihydrobiopterin or from DHNA itself since protein Raman bands are slightly affected by binding ligands (2). The major bands at 1482, 1559, 1605 and 1655 cm\(^{-1}\) in the Figure 3a spectrum can be assigned to the bound 7,8-dihydrobiopterin since corresponding bands in the solution spectrum of solution 7,8-dihydrobiopterin can be readily found (Figure 2a). The spectral features of the small band at 1679 cm\(^{-1}\) in Figure 3a and the dip at its low frequency side are likely due to incompletely nulled protein peaks in the difference spectrum. The 1679 cm\(^{-1}\)
peak does not shift upon $^{15}$N5 labeling of 7,8-dihydrobiopterin (Figure 3b) or deuteration of the sample (Figure 3c); thus it does not arise from the protonated C6=N5 stretch. It is interesting to note that the bands at 1482, 1559, and 1605 cm$^{-1}$ are all within 2 cm$^{-1}$ of their corresponding position in aqueous solution, thus, they can be assigned to the C7H2 bending, C=C stretching and C2=N1 stretching modes, respectively. The 1655 cm$^{-1}$ band in Figure 3a, assigned to the C6=N5 stretch based on its 18 cm$^{-1}$ down shift upon $^{15}$N5 labeling (Figure 3b), shifts up by 19 cm$^{-1}$ relative to its solution value (Figure 2a). Since this band remains at 1655 cm$^{-1}$ when the sample is deuterated (Figure 3c), N5 of 7,8-dihydrobiopterin is not protonated when bound to DHNA. Thus, on the basis of the Raman results, no significant amount of protonated N5 of bound 7,8-dihydrobiopterin can be detected under our experimental conditions. Similar experiments performed at pH 6.5 yield the same results (data not shown). We tentatively assign the 1615 cm$^{-1}$ band observed in Figure 3c to the C4=O stretch because it is near its position at 1623 cm$^{-1}$ found in the solution spectrum (Figure 2c) and has about the right Raman intensity for this band. The apparent small 7 cm$^{-1}$ downshift of the C4=O stretch upon binding is consistent with a small amount of bond polarization that is often observed for keto groups bound to proteins. Such a bond polarization comes about from increased hydrogen bonding in the protein relative to water (2).

In an attempt to understand the influence of the binding pocket on the C6=N5 stretch frequency, we have conducted additional ab initio calculations on a series of model systems of 7,8-dihydrobiopterin, including its complex with a carboxyl group in contact with N2H2 and N3H; a water molecule hydrogen bonded to the C=O or OH groups in various configurations; and different orientations of the OH group of the chain relative to the N5. The results show that the addition of a carboxyl group has a relative large effect on the C6=N5 stretch mode, causing a
shift of this mode by about 10 cm\(^{-1}\) but towards lower frequency. The hydrogen bonding on the OH or C=O by a water molecule does not have significant effect on the C6=N5 stretch, causing less than 5 cm\(^{-1}\) shift. On the other hand, the orientation of the first OH group of the arm on C6 has a significant effect on the C6=N5 stretch. When the orientation of this OH changes from antiparallel (relative to N5) to a syn conformation, the C6=N5 stretch is predicted to shift by about 10 cm\(^{-1}\) towards higher frequency.

**DISCUSSION**

The substrate binding site of DHNA near the pterin ring bears a strong similarity to the binding of folate substrates to dihydrofolate reductases. For example, the carboxyl of Glu74 in the DHNA binding pocket is positioned very similarly to that of the strictly conserved Asp group (Asp27 in *E. coli* DHFR) in the reductases. A structural water molecule is also found in *E. coli* DHFR (12) in some of the crystallographic structures in a place similar to that in the DHNA binding site (see scheme I). In the dihydrofolate to tetrahydrofolate reaction, N5 of the pterin ring is protonated. It is often supposed that the conserved Asp groups of DHFRs either donate the proton to N5 via active site structural water molecules or stabilizes the positive charge on protonated N5 (4,13,14). There is some evidence that the pterin ring C=O bond is polarized (5), and this is consistent with a proton relay system from Asp27 to N5 via binding site water molecule(s). It has been shown that the pKa of N5 of dihydrofolate bound to *E. coli* DHFR is raised from its solution value of 2.6 to 6.5, and this structural attribute can be a major factor in the activity of this enzyme. Asp27 is essential for enzymic activity in DHFR ([Howell, 1986 #361](#361)) as well as structurally necessary for raising the pKa of N5 *in situ* (6). Given these
structural similarities, it is reasonable to compare the structures of the bound pterin ring in DHFR to that in DHNA.

Our UV-Vis and Raman studies indicate that, in the Michaelis complex of 7,8-dihydrobiopterin/DHNA, more than 90% of N5 is unprotonated. Furthermore, there is no detectable change in its UV-Vis and Raman spectra when the sample pH is changed from 7.8 to 6.5, suggesting that no significant amount of protonated N5 is present. The results place the pKa of N5 of bound 7,8-dihydrobiopterin to be less than around 5. The kinetic data on the DHNA catalyzed reaction on 7,8-dihydrobiopterin shows that the optimal pH for the DHNA catalyzed reaction is about 9, similar to that for the 7,8-dihydronopterin, consistent with the argument that protonated N5 is not required in the ground state or in the transition state of the reaction. We also find that the C=O group of the pterin ring of bound 7,8-dihydrobiopterin is apparently not particularly polarized from its solution value. All of this is in contrast to DHFR where the pKa of N5 is 6.5 and the pH profile for hydride transfer has its half maximum also near this value (15,16). Thus the effect on the pterin ring by binding to DHNA is quite different than that found for dihydrofolates bound to (E. coli) DHFR despite the similarities in the structure of the binding pocket. The pterin ring binding pocket of DHNA would appear to be design for binding of the ring and not for promotion of the catalyzed reaction. This notion is strongly reinforced in studies of mutant DHNA whereby only a small decrease (to 25% of the wild type) in activity is found for enzymes that lack the active site Glu74 (unpublished data).

It needs to be pointed out here that our preliminary results suggest that the product from DHNA catalyzed conversion of 7,8-dihydrobiopterin is not the same as that from 7,8-dihydronopterin, the natural substrate of DHNA. Thus, the terminal OH group in 7,8-dihydronopterin plays a crucial role in the reaction, since its presence does not only dictate the reaction rate but also the reaction pathway. On the other hand, the unprotonated state of N5 in
7,8-dihydrobiopterin is unlikely the reason for the slow turnover rate compared to 7,8-dihydronopterin since both of these molecules have similar pH reaction profiles, as mentioned above.

The only significant observed change in the electronic structure of pterin ring of 7,8-dihydrobiopterin bound to DHNA relative to its solution form observed by our study is within the C6=N5 bond. The C6=N5 stretch increases 19 cm\(^{-1}\) upon binding. This is an unusual change and is difficult to bring about. As outlined in Results, our calculations indicate that none, i.e. Glu74, the active site water molecule, Lys100 (see Scheme I), of the active site polar groups can bring about the increased electron density in the C6=N5 bond through electrostatic interactions (i.e. hydrogen bonding) that would result in the increased frequency of the C6=N5 stretch. This is particularly surprising with regards to the hydrogen bonding of the NH\(_2\) group of Lys100 to substrate that certainly participates in enzymic catalyzed C-C bond breaking. This leaves steric interactions at the active site that change the conformation and electron distribution of the pterin ring through geometry changes and/or imposition of strain on the bound substrate. For example, there is a substantial upward shift in frequency (although only about half that observed) for a C6=N5 bond that is syn to the adjacent N6-C(OH)- group compared to the anti conformation (see Table 1). It is certainly possible that the enzyme imposes strain on the alcoholic alkane 'arm' of the bound biopterin which brings about sufficient electron delocalization to increase the electron density in the C6=N5 bond, hence increasing the C6=N5 stretch.
Figure Legend

Figure 1. UV-Vis absorption spectra of DHNA/7,8-dihydrobiopterin complex taken at 1, 10, 25, 55, 80, 120, 150, 180, and 300 min, respectively, after sample mixing. The sample was in 25mM tris 150mM NaCl at pH7.8 at 32°C. The concentration of DHNA/7,8-dihydrobiopterin was ~0.05 mM. The bound 7,8-dihydrobiopterin has a $\lambda_{\text{max}}$ at 333 nm, slightly red shifted from the 7,8-dihydrobiopterin in solution. The $\lambda_{\text{max}}$ of the product is at 426 nm.

Figure 2. Raman spectra of (a) 7,8-dihydrobiopterin in H$_2$O, (b) $^{(15N5)}$7,8-dihydrobiopterin in H$_2$O; (c) 7,8-dihydrobiopterin in D$_2$O. The concentration of the sample was 10 mM and the sample pH was 6.5. The spectrum of water was subtracted from these spectra.

Figure 3. Raman difference spectra between DHNA/7,8-dihydrobiopterin and DHNA. A small contribution from the enzyme product of 7,8-dihydrobiopterin was also subtracted from these spectra (see text for details). (a) in H$_2$O; (b) with $^{(15N5)}$7,8-dihydrobiopterin in H$_2$O; (c) in D$_2$O. The sample was prepared in 25 mM tris, 150mM NaCl at pH 7.8. The temperature was 4°C and the concentration of DHNA and 7,8-dihydrobiopterin was 4 mM(active site) and 4 mM respectively.
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a DHNA/H₂ Biopterin -DHNA

b DHNA/[¹⁵N⁵]H₂ Biopterin -DHNA

c DHNA/H₂ Biopterin -DHNA in D₂O

Deng Figure 3
Active site of DHNA

Scheme I

Deng Scheme I
7,8-dihydrobiopterin

Scheme II

Deng scheme II
A Vibrational structure of 7,8-dihydrobiopterin bound to dihydroneopterin aldolase
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