Observation of a Short, Strong Hydrogen Bond in the Active Site of Hydroxynitrile Lyase from *Hevea brasiliensis* Explains a Large pKₐ Shift of the Catalytic Base Induced by the Reaction Intermediate

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The hydroxynitrile lyase from *Hevea brasiliensis* (Hb-HNL) uses a catalytic triad consisting of Ser⁶⁰, His⁴⁷⁵, and Asp⁹⁰⁷ to enhance the basicity of Ser⁶⁰-O⁻ by abstracting a proton from the OH group of the substrate cyanohydrin. Following the observation of a relatively short distance between a carboxyl oxygen of Asp⁹⁰⁷ and the N₆ of His⁴⁷⁵ in a 1.1 Å crystal structure of Hb-HNL, we here show by ¹H and ¹⁵N-NMR spectroscopy that a short, strong hydrogen bond (SSHb) is formed between the two residues upon binding of the competitive inhibitor thiocyanate to Hb-HNL: the proton resonance of H-N⁶⁰(His⁴⁷⁵) moves from 15.41 ppm in the free enzyme to 15.35 ppm in the complex, the largest downfield shift observed so far upon inhibitor binding. Simultaneously, the D/H fractionation factor decreases from 0.98 to 0.35. In the observable pH range, i.e. between pH 4 and 10, no significant changes in chemical shifts (and therefore hydrogen bond strength) were observed for free Hb-HNL. For the complex with thiocyanate, the 19.35 ppm signal returned to 15.41 ppm at pH 8, which indicates a pKₐ near this value for the H-Ne₆(His⁴⁷⁵). These NMR results were analyzed on the basis of finite difference Poisson-Boltzmann calculations, which yielded the relative free energies of four protonation states of the His⁴⁷⁵-Asp⁹⁰⁷ pair in solution as well as in the protein environment with and without bound inhibitor. The calculations explain all the NMR features, i.e. they suggest why a short, strong hydrogen bond is formed upon inhibitor binding and why this short, strong hydrogen bond reverts back to a normal one at pH 8. Importantly, the computations also yield a shift of the free energy of the anionic state relative to the zwitterionic reference state by about 10.6 kcal/mol, equivalent to a shift in the apparent pKₐ of His⁴⁷⁵ from 2.5 to 10. This huge inhibitor-induced increase in basicity is a prerequisite for His⁴⁷⁵ to act as general base in the Hb-HNL-catalyzed cyanohydrin reaction.

Short, strong hydrogen bonds (SSHb) occur when the pKₐ of a hydrogen-bonded donor matches the one of the acceptor. If the hydrogen bond is sufficiently short, the central maximum of the (more or less symmetrical) hydrogen atom potential along the line connecting donor and acceptor may fall below the vibrational groundstate, resulting in a delocalized hydrogen atom. This situation is then referred to as a “low-barrier hydrogen bond” (LBHB). Low-barrier hydrogen bonds may occur in solutions and crystals of organic and inorganic compounds. Their observation in the active sites of enzymes (2–4) has led to controversy concerning their significance for the mechanism of enzyme catalysis (2–11). It has been suggested that the energy gained by the transient conversion of a “normal” hydrogen bond into a short, strong hydrogen bond can be used to stabilize transition states of enzymatic reactions (2–7). However, this energy gain (12) and its relevance for enzyme catalysis did not remain undisputed (8–11). There are indeed enzymes where a LBHB was observed that was shown not to be an inherent requirement for substantial rate enhancement (13). Irrespective of their energetic relevance, low-barrier hydrogen bonds are useful diagnostic tools to identify the matching pKₐs of a hydrogen bonded donor-acceptor pair, which was used to rationalize enzymatic mechanisms (14).

The occurrence of SSHbs has so far been reported for a considerable number of enzymes, including ketosteroid isomerase (15–17), triosephosphate isomerase (18), serine proteases (3, 6, 19), tryptophan synthase (20), 2-amino-3-ketobutyrate-CoA ligase (21), and cholinesterases (22, 23). Their occurrence has typically been suggested by NMR spectroscopy (24, 25). The exposure of the delocalized proton decreases the electron density around the proton nucleus, which shifts the NMR signal to very low field (higher frequency; typically 18–22 ppm (26)). Another NMR-observable quantity used to identify a short, strong hydrogen bond is the fractionation factor φ of the associated proton, defined as the equilibrium constant for the exchange of hydrogen by deuterium with the solvent (27). φ depends on the relative strength of a hydrogen bond compared with the solvent, with values lower than 1 indicating stronger hydrogen bonds (24).

Hydroxynitrile lyase (HNL) (EC 4.1.2.39) catalyzes the...

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1. The abbreviations used are: SSHb, short, strong hydrogen bond; LBHB, low-barrier hydrogen bond; HNL, hydroxynitril lyase; HbHNL, HNL from *Hevea brasiliensis;* NMR, nuclear magnetic resonance; FDPB, finite difference Poisson-Boltzmann.
2. The terms "short, strong hydrogen bond" and "low-barrier hydrogen bond" are being used almost synonymously in the literature.
cleavage of cyanohydrins to hydrocyanic acid plus the cor-
responding aldehyde or ketone (Fig. 1A). The release of HCN
serves as a defense against herbivores and microbial attack for
a variety of plants (28–30). In aqueous solution, cyanohydrin
cleavage occurs spontaneously above pH 5, whereas the en-
zyme reaction also occurs at lower pH values (down to pH 3;
Ref. 29). This is technologically exploited for the enantioselective
synthesis of chiral cyanohydrins (31–35), making use of the
reverse in vivo reaction. Structurally, the HNL, from the trop-
ical rubber tree Hevea brasiliensis (HbHNL) belongs to the
α/β-hydrolase superfamily (36) and has a prototypical catalytic
triad consisting of residues Asp207, His235, and Ser80. However,
the environment of this triad differs from other hydrolases.

![Figure 1A](image1.jpg)

**Fig. 1.** A, the chemical reaction catalyzed by hydroxynitrile lyases. B, proposed mechanism of the reaction catalyzed by HbHNL, formulated for the cyanohydrin cleavage direction.

Here we report NMR experiments that show that this “catal-
lytic” hydrogen bond converts into a short, strong hydrogen
bond upon addition of a strong competitive inhibitor mimicking
the rate-limiting transition state of the enzyme reaction. To
rationalize this NMR result, we performed Poisson-Boltzmann
computations, which yield a consistent explanation for the
occurrence of the SSHB. In fact, the results of the computations
show how the occurrence of the SSHB is paralleled by a cata-
lytically relevant increase in basicity of the general base upon
approach of the transition state.

### EXPERIMENTAL PROCEDURES

**Materials—**Crude natural abundance HbHNL was obtained by
Roche Applied Science in a 20 mM citrate phosphate buffer pH 6.5 (5300
units/ml and 59 mg/ml) and purified as reported (45). For NMR meas-
urements the protein was dialyzed against 50 mM potassium phosphate
buffer, pH 7.4. For the isotopically labeled protein, 15N-ammonium
sulfate, 13C-glycerol (Martek, Columbia, MD), and 13C-methanol (Cam-
bridge Isotope Laboratories, Andover, MA) were used for the minimal
medium. Anhydrous potassium thiocyanate (KSCN), acetone, benza-
dehyde, mandelonitrile, HCl, sodium hydroxide, and deuterium oxide
(D2O, 99.8% at % D) were purchased from Merck. All solvents and
reagents were analytical or reagent grade and were used without fur-
ther purification.

**Enzyme Preparation—**Wild-type HbHNL was labeled with 15N or 15N
and 13C by expression from the Pichia pastoris strain 2386 GS115
H′ M pHLD1.17-HNL (Technical University Graz) in a minimal me-
dium that has been slightly modified from the published fermenta-
tion procedure (45). Instead of a bioreactor, shake flasks containing 0.4 M
KH2PO4/K2HPO4, pH 5, 0.1 g (15NH4)2SO4 per 200 ml minimal medium
were used. In addition, the concentrations of trace elements were lower
by a factor of four. The protein was purified by anion exchange (HiTrap
Q HP, Amersham Biosciences) and gel filtration chromatography (Su-
perdex200, Amersham Biosciences) as reported previously (45). All
samples were >95% pure based on SDS-PAGE analysis. Protein con-
centration was determined by the BCA assay (Bio-Rad). The 15N Hb-
HNL sample was concentrated to 10 mg/ml (activity ~ 200 units/ml) and
the 15N/13C double labeled HbHNL to 19 mg/ml with an activity of
321 units/ml.

**Hydroxynitrile Lyase Activity Test—**Enzyme activity (39) was meas-
ured following the cleavage of racemic mandelonitrile into benza-
dehyde and HCN, at a substrate concentration of 12 mM and at an enzyme
concentration of about 0.74 µg/ml. The assay was performed in 20 mM
glutamate buffer at pH 5 and 25 °C, and the formation of benza
dehyde...
was monitored spectrophotometrically at 280 nm. In addition to the enzymatic reaction the base-catalyzed chemical reaction has to be measured separately and subtracted. The reaction was monitored for 2 min, and the enzyme activity was calculated from the slope in a plot of \( \Delta \text{R} \) versus time.

**NMR Sample Preparation**—For all measurements 600 \( \mu \)l of between 0.33 and 0.63 mm HNL in 50 mm potassium phosphate buffer, pH 7.4, 90:10% \( H_2O/D_2O \) were used. In the titrations with acetone, benzalde- hyde, and thiocyanate, concentrations of 1–50 mm, 1–6 mm, and 0.2–1 mm were used, respectively. For the determination of the fractionation factors we used \( H_2O/D_2O \) buffer mixtures of 550:50 \( \mu \)l, 420:180 \( \mu \)l, 300:300 \( \mu \)l and 180:420 \( \mu \)l. The pH value was changed within the range pH 4–10 for the acid/base titrations. Starting from a sample with pH 7.0, the titrations involved steps of 3 to 5 \( \mu \)l of 0.1 HCl or 0.1 NaOH. The pH in the 5 mm NMR sample tube was monitored using an Orion Model 9803 NMR pH electrode.

**NMR Experiments**—All one-dimensional \( ^1H \)-NMR spectra were collected on a Bruker Avance DRX 500 MHz NMR spectrometer, where the water signal was suppressed using the WATERGATE sequence (46). Due to the fast relaxation of the protein signals, at least 1024 scans had to be acquired for each one-dimensional spectrum consisting of 16,000 data points. A 60° phase-shifted squared sine-bell window function was applied prior to Fourier transformation. The \( ^1H \)-\( ^15N \) HSQC was ac-

The fractionation factor \( \phi \) is defined as the equilibrium constant of the reaction (27) as seen in the Equation 1 as follows.

\[
En - H + D_{aq} \rightleftharpoons En - D + H^n \quad \phi = \frac{[En - D][H^n]}{[En - H][D_{aq}]} \quad (Eq. 1)
\]

By substituting the concentration of \( En - H \) with the directly propor-
tional signal intensity I and expressing the \( [H^n]/[D_{aq}] \) ratio with X, the mole fraction of \( H^n \) in the used \( H_2O/D_2O \) mixture, this equation can be rearranged as shown in Equation 2.

\[
I = \frac{I_{max} \cdot X}{\phi \cdot (1 - X) + X} \quad (Eq. 2)
\]

Here, \( I_{max} \) is the maximal peak intensity at X = 1.00 (24). The fractionation factor \( \phi \) was calculated from the slope of a straight line obtained by least squares fitting the reciprocal measured signal intensities (1/I) as a function of \( (1 - X)/X \) in the linearized form of Equation 2 as follows.

\[
\frac{1}{I} = \frac{\phi \cdot (1 - X)}{I_{max} \cdot X} + \frac{1}{I_{max}} \quad (Eq. 3)
\]

**Calculation of Electrostatic Interactions**—Electrostatic interaction and solvation-free energies were calculated using the finite-difference-Poisson-Boltzmann (FDPB) method as implemented in the program DelPhi (47). The two values for the bulk dielectric constant were set to 4.0 (protein) and 80.0 (solvent), and an ionic strength of 0.145 m was assumed throughout. The scale of the grid was 2 Å. The ion exclusion radius was set to 2 Å and the probe radius (for surface calculations) to 1.4 Å. All calculations were performed with the eight structures of \( HbHNL \) currently available in the Protein Data Bank (codes: 1q4, 1yas, 2yas, 3yas, 4yas, 5yas, 6yas, and 7yas) (36, 37, 43), and the resulting energy values were averaged. Water molecules and ligand atoms present in the structures were removed. In the case of alternate conforma-
tions, the higher occupied conformer was kept. Aspartate (except Asp207), glutamate, arginine, and lysine side-chains were modeled as charged. Tautomers and protonation states of histidine residues (except His(235)) were assigned by inspection.

A special situation was encountered for the cationic pair, in which the aspartate is neutral but still acts as hydrogen bond acceptor. For the protein-bound pair, on either of the two carboxyl oxygen a proton would not find a suitable hydrogen bond acceptor, but possibly lead to unac-
ceptable interactions with the protein (main-chain amide nitrogen at-
oms of Ile(209) and Phe(210), side-chain of Asn(104)). Neutralization of the Asp(207) side-chain was therefore accomplished by modifying the charges only, without the addition of a hydrogen atom.

For the FDPB-calculations in water, the entire amino acids for His(235) and Asp(207) were used in a conformation as observed in the tructure of \( HbHNL \) crystal structure (36, 37, 43) and the obtained energy values for the various structures were averaged as described for the calculations in the protein environment.

**RESULTS**

**Observation of a Downfield-shifted \(^1H\)-NMR Signal**—The observation of a short distance (2.67 Å) between Oe(Asp(207)) and Nδ(His(235)) in the high-resolution crystal structure of \( HbHNL \) (43) indicated the possibility for a short, strong hydrogen bond in the active site of this enzyme. A \(^1H\)-NMR spectrum was acquired between –3 to 22 ppm, and a signal was indeed observed at 15.41 ppm (Fig. 2). Because the large molecular size (60 kDa) of this homodimeric (48) protein prohibited a complete sequential NMR assignment, we had to resort to indirect ways to corroborate the assignment of the observed signal at 15.41 ppm to the proton attached to Nδ (His(235)).

**Signal Assignment**—The following empirical correlation be-
tween proton chemical shifts and hydrogen bond lengths in imidazolium-carboxylate complexes (23) is as follows

\[
D = 1.99 + 0.198 \ln(\delta) + (10.14/\delta)^3 \quad (Eq. 4)
\]

was used to deduce a heteroatom separation from the observed chemical shift and compare it with the cry1tallographically observed value for the Nδ(His(235))—Oe(Asp(207)) distance. The distance D obtained from the proton chemical shift \( \delta \) = 15.41 ppm is 2.65 Å, in good agreement with the crystallographically observed (43) distance of 2.67 Å.

To ensure that the downfield signal belongs to a proton attached to a nitrogenous amino acid, a \(^1H\)-\(^15N\)-HSQC was acquired on a sample of 0.33 mm \(^15N\)-isotopically enriched \( HbHNL \). The proton and nitrogen carriers were placed at 15.41 and 190 ppm, respectively, to optimize the signal intensities in the region of the 15.41 ppm signal. The resulting two-dimensional spectrum is shown in Fig. 3, where the signal at 15.41 and 180.08 ppm is clearly visible. Attempts to correlate this signal to a nearby \(^13C\) nucleus in \(^14N\)\(^13C\) doubly labeled \( HbHNL \) in a two-dimen-
sional H-(N)-C correlation experiment (49) were unsuccessful, presumably due to the fast relaxation within this large protein and to the relatively small \(^1J_{NC}\) coupling constants within histidine side-chains (50).

The assignment of the 15.41 ppm signal was further con-
firmed by determining the shift induced by the presence of benzaldehyde in the active side. The crystal structure of \( HbHNL \) complexed with benzaldehyde has not yet been reported, but modeling studies (38) yield a stable solution which shows the aromatic ring 7.1 Å away from the N=N(His(235)), as shown in Fig. 4A. This orientation was used to calculate (program SHIFTTS (51)) the change in chemical shift of the proton at-
tached to Nδ(His(235)) induced by the ring current of benzalde-
hyde. The computed shift of –0.13 ppm compares well with the experimentally observed shift of the proton signal from 15.41 ppm to 15.22 ppm upon titrating 6 mm benzaldehyde into a solution of 0.6 mm \( HbHNL \) (Fig. 2C). In a control experiment, acetone was titrated into a solution of 0.6 mm \( HbHNL \) up to a
concentration of 50 mM, which did not cause any shift of the downfield signal (data not shown).

The Effect of the inhibitor Thiocyanate and of the Substrate Mandelonitrile—

Although a chemical shift of 15.41 ppm for a H-N\(^{1}\) (His\(^{235}\)) is clearly outside the "regular" range (10.1 ± 3.5 ppm, BioMagResBank, www.bmrb.wisc.edu (52)), it is not yet a low barrier hydrogen bond, whose \(^{1}\)H-NMR signal is expected between 18–22 ppm (26). Because short, strong hydrogen bonds are often observed in protein conformations mimicking a transition state, we also titrated the strong inhibitor thiocyanate (\(K_{i} = 5.5\) mM (37, 53)) into a solution of 0.5 mM HbHNL. Upon addition of thiocyanate, the signal at 15.41 ppm disappears and a new signal appears at 19.31 ppm (Fig. 5), i.e. at a value characteristic for low barrier hydrogen bonds.

A different behavior is observed when adding mandelonitrile, one of the substrates of HbHNL (\(K_{m} = 3\) mM (39)). Upon titration of 0.1–3 mM mandelonitrile into a solution of 0.5 mM HbHNL, the signal at 15.41 ppm disappears and no signal beyond 13 ppm is observable. We assume that the dynamics of the HNL reaction prohibit the observation of H-N\(^{1}\) (His\(^{235}\)), whose resonance shifts to several different positions during one reaction cycle.

Hydrogen-Deuterium Fractionation Factors—

We have determined the fractionation factors of unliganded HbHNL and of HbHNL complexed with SCN\(^{-}\) by monitoring the intensities of the downfield-shifted signal in varying ratios of D\(_{2}\)O/H\(_{2}\)O. The reciprocal normalized intensities \(1/I\) plotted versus \((1/X)/X\) are shown in Fig. 6 for HbHNL and HbHNL-SCN. From the slope of this normalized plot, a fractionation factor of 0.98 is obtained for HbHNL in the absence of thiocyanate and of 0.35 in the presence of 1 mM thiocyanate. The latter value is typical for a short, strong hydrogen bond.

pH Titration—

The protonation properties of His\(^{235}\) were investigated by pH titrations in the range between pH 4 and 10 for HbHNL in the presence and in the absence of SCN\(^{-}\). These studies were complicated by the pH instability of the protein. In the uncomplexed form, HbHNL starts to precipitate below ~pH 5, and it denatures above pH 10 as indicated by the complete collapse of the one-dimensional \(^{1}\)H spectrum to random-coil values (54).

Between ~pH 4 and 9, titration of free HbHNL did not lead
to detectable spectral changes. In the presence of thiocyanate, H6HNL appears to be slightly more stable at lower pH values, as judged from the later onset of precipitation, which now occurs below pH 4. Although at the low pH side, no pH-dependent spectral changes are observed, the signal at 19.35 ppm starts to diminish above pH 7 with concomitant reappearance of the 15.41 ppm signal in the presence of thiocyanate. This process is complete at pH 9.

Poisson-Boltzmann Calculations—FDPB calculations were used to estimate the relative free energies of the four protonation states of the His$^{235}$–Asp$^{207}$ diad shown in Fig. 7A. These states included a zwitterionic state (arbitrarily defined as the...
The fractionation factor equals the slope of the least square fit.

The standard deviations and least square fits. In these plots the fractionation factor equals the slope of the least square fit.

The negative charge of Asp207 in the anionic state raises the pK values 1/H9004 and, for the transitions from zwitterionic and neutral is inherently pH-independent as it only involves proton transfer among the two residues, the zwitterionic → cationic and zwitterionic → anionic transitions involve proton exchange with solvent and therefore depend on the pH of the solution.

The estimation of relative free energy values of the different protonation states of the diad within the protein was accomplished through three sets of thermodynamic cycles. One of them is shown in Fig. 7B. They enabled the calculation of ΔG-values solely from the pK values of histidine and aspartic acid in solution plus two parameters that are accessible by FDPB methods. They are the electrostatic interaction energy of the two residues in the hydrogen-bonded pair and the solvation free energy of the diad for the transfer from water into the protein environment.

Each thermodynamic cycle starts from the two residues in water at infinite separation, where the relative free energies ΔG0 depend only on the pK values of the two residues (6.5 for His and 4.4 for Asp (55)) and, for the transitions from zwitterionic to cationic or anionic state, on the solution pH as shown in the following equation.

\[ \Delta G_{H^+} = 2.3RT(pK_{His} - pK_{Asp}) \]
\[ \Delta G_{\text{anion}} = 2.3RT(pK_{\text{anion}} - pH) \]
\[ \Delta G_{\text{cation}} = 2.3RT(pH - pK_{\text{Asp}}) \]

The approach of the two solvated groups results in the formation of different types of hydrogen bonds (neutral H-bond or salt bridge). Thereby the electrostatic interaction of His\textsuperscript{235} with the negative charge of Asp\textsuperscript{207} in the anionic state raises the pK\textsubscript{a} of the former. Likewise, the pK\textsubscript{a} of Asp\textsuperscript{207} is lowered by the same amount due to the interaction with the positive charge of His\textsuperscript{235} in the cationic state. These shifts correspond to differences in electrostatic interaction energy ΔΔG\textsubscript{elec} with the inherent symmetry of these mutual interactions (causing equal pK\textsubscript{a} shifts in opposite directions) causing a factor of two in the equation for ΔG\textsubscript{H+}.

\[ \Delta G_{\text{neutral}} = \Delta G_{\text{neutral}} + 2\Delta G_{\text{elec}} \]
\[ \Delta G_{\text{anion}} = \Delta G_{\text{anion}} + 2\Delta G_{\text{elec}} \]
\[ \Delta G_{\text{cation}} = \Delta G_{\text{cation}} + 2\Delta G_{\text{elec}} \]

The FDPB calculations yielded an average value for ΔΔG\textsubscript{elec} of 1.1 (±0.1) kcal/mol.

The final step in each thermodynamic cycle consists of the transfer of the His-Asp diad from water into the protein (Fig. 7B).

\[ \Delta G_{\text{neutral}} = \Delta G_{\text{neutral}} + \Delta G_{\text{elec}} \]
\[ \Delta G_{\text{anion}} = \Delta G_{\text{anion}} + \Delta G_{\text{elec}} \]
\[ \Delta G_{\text{cation}} = \Delta G_{\text{cation}} + \Delta G_{\text{elec}} \]

ΔΔG\textsubscript{elec} values were calculated using FDPB methods for the free HNL enzyme and for the complex with the micromolar inhibitor thiocyanate (Fig. 4B and Ref. 37). For the free enzyme, we obtained −8.3 (±0.3) kcal/mol for ΔΔG\textsubscript{elec}, −9.9 (±0.7) kcal/mol for ΔΔG\textsubscript{anion} and 7.4 (±0.5) kcal/mol for ΔΔG\textsubscript{cation}. The corresponding values for the thiocyanate complex were −4.5 (±0.5) kcal/mol (neutral), 3.9 (±1.3) kcal/mol (anionic), and 3.7 (±0.5) kcal/mol (cationic).

The pH dependence of the relative free energies of the diad are shown in Fig. 8 for the protein environment. For the calculation of ΔG2 the pH dependence of ΔΔG\textsubscript{elec} was ignored, i.e. ΔΔG\textsubscript{elec} was calculated neglecting the pH dependence of the charges of surrounding amino acid residues. This approximation was considered appropriate for the pH range of about 4–9, which was used in the NMR titration experiments.

DISCUSSION

The above NMR evidence leaves no doubt that the proton between the two active-site residues His\textsuperscript{235} and Asp\textsuperscript{207} gives rise to a downfield-shifted NMR signal, which appears at 15.41 ppm at all accessible pH values in the absence of inhibitor. In the presence of the strong inhibitor thiocyanate, the signal shifts to 19.35 ppm, and the D/H fractionation factor of 0.35 classifies it as originating from a proton involved in a short, strong hydrogen bond. This short, strong hydrogen bond persists at low pH, but reverts back to a normal H-bond (with the signal at 15.41 ppm) under basic conditions (pH > 8). Similar results were reported for acetylcholinesterase (22), α-chymotrypsin (10), and chymotrypsin (3), but with considerably smaller inhibitor-induced shifts (about 3 ppm versus 3.8 ppm) and with a different pH dependence.

These observations can be rationalized on the basis of free energy calculations for a small number of protonation states. Such estimates of free energies (56) and similar continuum solvent approaches have successfully been used to compute pK\textsubscript{a} shifts of residues in proteins as well as solvation free energies (57). The calculations yield ramifications for the mechanism of the HbHNL-catalyzed cyanohydrin reaction, which are discussed below.

Free Energy Calculations—The catalytic diad (consisting of residues His\textsuperscript{235} and Asp\textsuperscript{207}) comprises at least 4 distinct but coupled protonation sites. A discussion of its pH- and inhibitor-dependent protonation behavior in terms of individual pK\textsubscript{a} values for each site is complicated because the pK\textsubscript{a} of each site depends on the state of protonation of the other sites. In other words, there are 16 formally different ways how a system of 4 coupled sites can be protonated. Not all of these possibilities are equally probable.

To simplify the problem, we have selected four protonation states of the His\textsuperscript{235}-Asp\textsuperscript{207} pair on the basis of chemical plausibility, as defined in Fig. 7A. Using an appropriately chosen system of thermodynamic cycles in combination with finite difference Poisson-Boltzmann calculations, the relative free energies of these four protonation states of the His\textsuperscript{235}-Asp\textsuperscript{207} diad were estimated as a function of pH for HbHNL as observed crystallographically in the absence (Fig. 8A) and in the presence...
ence (Fig. 8B) of thiocyanate. To our knowledge, this constitutes a novel approach for the quantitative analysis of short, strong hydrogen bonds in proteins, although similar cycles have been used qualitatively to analyze low-barrier hydrogen bonds in proteins (8, 9) and in the gas phase (58). Most importantly, the use of such cycles and the treatment of the His-Asp diad as an entity does away with the difficulties in discussing distinct pKₐ values of the components of the diad.

The titration curves for the His-Asp diad in HbHNL (Figs. 8, A and B) show that for the free enzyme the anionic state has the lowest free energy except at very low pH. This is in full agreement both with NMR (one signal at 15.41 ppm between pH 4 and 9) and high-resolution crystallographic evidence (density originating from one proton located near Nδ of His235).

Among the two states whose relative free energy is pH-independent, the neutral state is lower than the zwitterionic state as a result of the low dielectric constant of the medium and the interaction of the zwitterionic form with the positive charge of
which agrees well with a point of intersection of anionic and neutral states (Fig. 8A). The quality of the calculations can be estimated for the anionic state. Thus, within the accuracy of the FDPB calculations, the theory provides a perfect rationalization of the SSHB signal at 19.35 ppm reverts to the 15.41 ppm signal of the latter is indeed observed at all but very high pH, where the pKa of His235 amounts to about 2.5 as judged from NMR experiments. At very low pH the neutral state would be favored, but its observation is prevented by enzyme instability. The cationic state is energetically unfavorable at all pH values.

The (delocalized) negative charge of the thiocyanate inhibits the catalytic base. Similar effects also have to be relevant for other enzymes with general acid/base catalysis. We wish to emphasize, however, that our results neither imply nor exclude the factors enabling proton abstraction from the substrate by the catalytic base. The (delocalized) negative charge on the substrate upon deprotonation and substrate-free form high enough to deprotonate the cyanohydrin OH (with a pKa of around 10.7 (59)), it would become protonated by solvent. In fact, a substrate-induced pKa-shift is exactly what is implied by the above free energy computations and the NMR results. Concomitant with the inhibitor-induced equalization of the energy levels of zwitterionic and neutral protonation states (leading to the observation of a SSHB), the free energy of the anionic state is increased by about 10.6 kcal/mol (i.e. the apparent pKa of His235 shifts from 2.5 to 10). Insofar as thiocyanate mimics the transition state (see above), this amounts to an increase in basicity of the catalytic base upon approach of the transition state, brought about by the nascent negative charge on the substrate upon deprotonation and subsequent cleavage of the cyanohydrin with formation of a (also negatively charged) cyanide ion. These observations parallel the ones made for ketosteroid isomerase (17).

We showed that a SSHB forms at or near the transition state of the rate-determining step of the enzyme-catalyzed cyanohydrin cleavage, which we consider relevant for understanding the factors enabling proton abstraction from the substrate by the catalytic base. Similar effects also have to be relevant for other enzymes with general acid/base catalysis. We wish to emphasize, however, that our results neither imply nor exclude the possibility for extra stabilization of the transition state due to the formation of a SSHB.

**Mechanistic Implications**—His235 acts as the general base in HbHNL, because it effects (via Ser30) the crucial step of cyanohydrin deprotonation. This step has to involve a substrate-induced pKa-shift of its side-chain, because in the substrate-free form of the enzyme, the His235 side-chain is in partial contact with solvent and, if its pKa were already in the substrate-free form high enough to deprotonate the cyanohydrin OH (with a pKa around 10.7 (59)), it would become protonated by solvent. In fact, a substrate-induced pKa-shift is exactly what is implied by the above free energy computations and the NMR results. Concomitant with the inhibitor-induced equalization of the energy levels of zwitterionic and neutral protonation states (leading to the observation of a SSHB), the free energy of the anionic state is increased by about 10.6 kcal/mol (i.e. the apparent pKa of His235 shifts from 2.5 to 10). Insofar as thiocyanate mimics the transition state (see above), this amounts to an increase in basicity of the catalytic base upon approach of the transition state, brought about by the nascent negative charge on the substrate upon deprotonation and subsequent cleavage of the cyanohydrin with formation of a (also negatively charged) cyanide ion. These observations parallel the ones made for ketosteroid isomerase (17).

We showed that a SSHB forms at or near the transition state of the rate-determining step of the enzyme-catalyzed cyanohydrin cleavage, which we consider relevant for understanding the factors enabling proton abstraction from the substrate by the catalytic base. Similar effects also have to be relevant for other enzymes with general acid/base catalysis. We wish to emphasize, however, that our results neither imply nor exclude the possibility for extra stabilization of the transition state due to the formation of a SSHB.
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Observation of a Short, Strong Hydrogen Bond in the Active Site of Hydroxynitrile Lyase from *Hevea brasiliensis* Explains a Large pKₐ Shift of the Catalytic Base Induced by the Reaction Intermediate
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