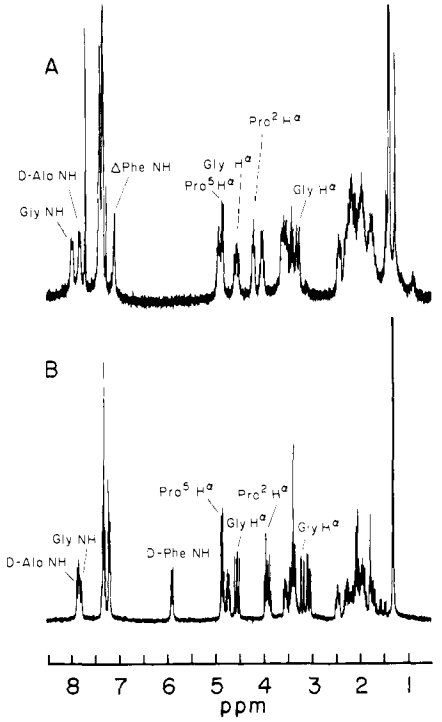

Figure 1. ^{1}H NMR spectra (250 MHz): (A) *cyclo*(Gly^1-Pro^2-Δ^z-Phe^3-D-Ala^4-Pro^5) in $CDCl_3$, 64 scans, concentration 11 mM; (B) *cyclo*(Gly^1-Pro^2-D-Phe^3-D-Ala^4-Pro^5) in $CDCl_3$, 16 scans, concentration 21 mM. All spectra were recorded at ambient temperature.

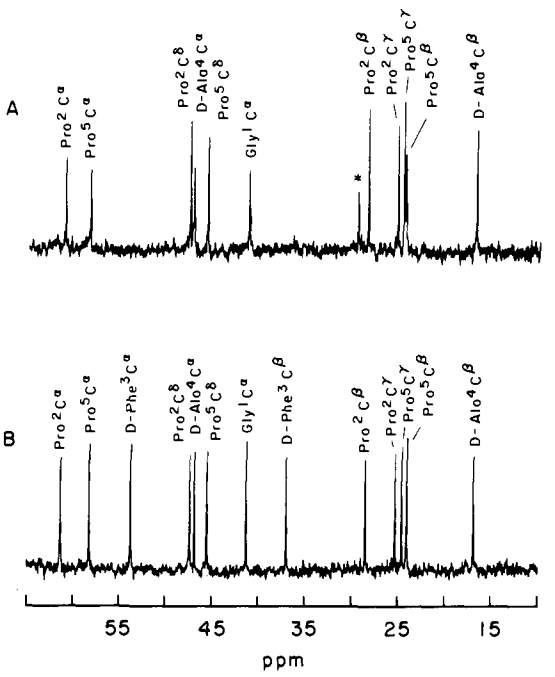

Figure 2. ^{13}C NMR spectra (62.9 MHz): (A) *cyclo*(Gly^1-Pro^2-Δ^z-Phe^3-D-Ala^4-Pro^5) in $CDCl_3$, 82000 scans, concentration 11 mM; (B) *cyclo*(Gly^1-Pro^2-D-Phe^3-D-Ala^4-Pro^5) in $CDCl_3$, 48000 scans, concentration 21 mM. All spectra were recorded at ambient temperature. The starred resonance in (A) is due to an acetone impurity.

ature coefficients (3.4 and 1.1 ppb/deg, respectively) relative to the exposed D-Phe NH (8.2 ppb/deg) at higher peptide concentrations (170 mM in $CDCl_3$ with 4% v/v dimethyl-d_6 sulfoxide).[15] The Pro^5 H$^\alpha$ resonance is shifted strongly to low field (4.85 ppm) and appears as a doublet with one large and one small coupling constant to the β protons, while the Pro^2 H$^\alpha$ appears as a triplet, i.e., nearly equal coupling constants at 3.94 ppm.[16] The downfield position and doublet appearance are typical of prolines occurring as the $i + 1$th residue of γ turn.[9,10] One of the proline C$^\beta$ resonances shows an upfield shift indicative of a γ turn (C$^\beta$ at 24.4 ppm, by comparison with the more usual 28–30 ppm).[9,10] The Pro carbon chemical shifts also confirm that II adopts an all-trans conformation.[17] These constraints uniquely determine the conformation of II.

Substitution of Δ^z-Phe for D-Phe (peptide I) causes very little change in the NMR spectral parameters (Figures 1 and 2): the D-Ala and Gly NH's are at low field (7.82 and 7.98 ppm) and have small temperature coefficients (2.1 and 1.7 ppb/deg) relative to the Δ^z-Phe NH (7.15 ppm, 4.6 ppb/deg); Pro^5 has a downfield "doublet" H$^\alpha$ resonance (4.85 ppm) and Pro^2 has a more usual position (4.25 ppm) and distorted triplet shape;[16] the proline β and γ carbon chemical shifts are nearly the same as in the peptide. These highly diagnostic indicators of backbone conformation argue strongly for the retention of the β,γ-turn conformation in the dehydropeptide. Even more striking are the detailed aspects of the ^{1}H NMR spectra: the chemical shifts vary little between I and II despite the presence of the Δ^z-Phe residue.

Thus, a Δ^z-Phe residue can be substituted for a saturated residue with minimal perturbation to a peptide's backbone conformation if it is placed in the $i + 2$ position of a type II β turn where ϕ and ψ angles are generally near 80° and 0°.[9] In a bioactive peptide, an analogous substitution of Δ^z-Phe should not cause loss of activity, provided that (1) the available backbone conformation coincides with the functional conformation and (2) the side-chain conformation at the substituted site is unchanged or not essential for activity.

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Crystallization-Induced Changes in Protein Structure Observed by Infrared Spectroscopy of Carbon Monoxide Liganded to Human Hemoglobins A and Zurich

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The relationship of a protein structure obtained by crystallography to the in vivo solution structure is of great interest to the study of protein structure and function. Upon crystallization, the structure of a protein molecule may change due to crystal lattice forces and different hydration levels.[2] With hemeproteins, ligand infrared spectra can provide a sensitive probe for monitoring ligand site structures in both crystals and solutions.[3,4] The spectra for CO, CN^-, N_3^-, NO, and O_2 as ligands have been observed for several hemeproteins in solution and provide evidence that multiple ligand site structures (conformers) are present.[5] IR

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Table I. Parameters of Deconvoluted C-O Stretch Bands for Infrared Spectra of Hemoglobin Carbonyls in Crystals and in Solution

		conformer[a]								
		CII[b]			CIII			CIV[b]		
hemoglobin carbonyl	temp, °C	ν_{CO}, cm^{-1}	$\Delta\nu_{1/2}$, cm^{-1}	% total area	ν_{CO}, cm^{-1}	$\Delta\nu_{1/2}$, cm^{-1}	% total area	ν_{CO}, cm^{-1}	$\Delta\nu_{1/2}$, cm^{-1}	% total area
HbACO[c]										
crystals	30	1943.0	8.0	3.4	α 1949.9	8.0	45.2	1969.0	8.0	6.9
					β 1954.6	7.8	43.1			
							88.3			
solution	30	1943.5	7.0	3.3	$\alpha + \beta$ 1951.2	8.0	92.9	1969.0	9.0	2.5
α subunit solution[d]	4	1944.0	7.5	5.0	α 1950.5	7.2	92.4	1969.0	9.0	1.0
β subunit solution[d]	4		e		β 1951.6	7.2	99.0	1969.0	9.0	0.8
HbZhCO[f]										
crystals	30	1943.5	8.6	4.4	α 1951.1	8.8	46.7	1969.7	7.1	7.8
					β 1959.5	8.4	40.1			
							86.8			
crystals	4	1945.0	8.0	4.2	α 1951.0	8.4	47.2	1969.8	6.8	4.0
					β 1959.5	8.1	43.9			
							91.1			
solution	30	1943.0	9.0	7.1	α 1950.0	7.4	46.9	1969.1	8.0	7.4
					β 1958.2	7.9	36.9			
							83.8			
solution	4	1942.5	7.0	4.4	α 1949.8	7.3	50.3	1969.3	9.0	4.8
					β 1958.3	7.6	39.0			
							89.3			

[a] Spectra were deconvoluted with a minimum number of symmetric curves defined between the limits of pure Gaussian to Lorentzian function as described in ref 11. Best fits were obtained with a minimum of four bands at 65% and 60% Gaussian nature for solution and crystals of HbACO and 75% and 70% for solution and crystals, respectively, of HbZhCO. Conformer CI at 1935 cm^{-1} constitutes 1% or less of the total area and is not included. [b] The minor conformers CII and CIV cannot be accurately separated into contributions from either α or β subunits. [c] HbA was purified by the method of ref 12 and stripped of organic phosphates by the method of ref 13. Crystals were prepared under a CO atmosphere at pH 6.7 according to ref 8. [d] Data from ref 9. [e] An insignificant percentage of the total area was observed in the CII region for separated β subunits. [f] HbZhCO was purified by the method of ref 14 and crystallized as described in ref 7.

spectra for carbonyl ligands of human hemoglobin A CO and hemoglobin Zurich CO (β63 His→Arg) in solution exhibit multiple C–O stretch bands.[5c] As found for myoglobin carbonyl, each Hb subunit may exhibit four discrete, rapidly interconverting conformers (CI, CII, CIII, and CIV).[5d] However, crystal structure studies for HbACO and HbZhCO consider only one conformer.[6,7] Here, carbonyl IR spectra of the two Hbs in crystals are shown to be somewhat different from solution spectra even though similar multiple conformers are present in both crystals and solution.

Each HbCO was crystallized as tetragonal bipyrimidal crystals (space group $p4_12_12$)[8] and contained less than 5% metHb as observed by visible spectroscopy on dissolved crystals. Crystallization of HbACO slightly shifted ν_{CO} and increased the width of the major CIII band and intensified the minor CIV band (Figure 1, Table I). In solution, the CIII band can be deconvoluted satisfactorily with only one theoretical curve whereas in the crystal at least two curves are required for adequate deconvolution (Figure 1). The two curves are similar in intensity and width to the CIII bands of separated α and β subunits in solution but the ν_{CO} values differ by 4.7 cm^{-1} in crystals vs. 1.1 cm^{-1} in solution (Table I).[9] Thus, the subunit differences in IR spectrum (and structure) are greater in the crystal. The spectrum supports a greater change in structure for the β subunits upon crystallization. Crystallization also perturbs the relative stabilities of the conformers; CIV increases nearly 3-fold as β CIII decreases. For HbACO in solution, a comparable enhancement in CIV has only been observed at pH <5 or >11 and temperature >45 °C.[5c] Although Hbs from many species exhibit spectra with a major

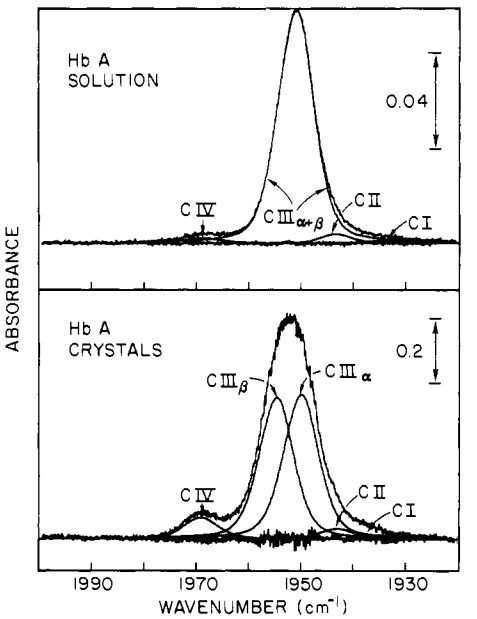

Figure 1. Infrared spectra of HbACO in solution and in crystals. Upper: HbACO (5mM) in 20 mM $NaPO_4$ buffer pH 7.2 at 30 °C. Lower: HbACO crystals bathed in 2.55 M $NaPO_4$ crystallization buffer pH 6.7 at 30 °C. Spectra were recorded in a Perkin-Elmer 180 spectrophotometer interfaced to a Tektronix 4051 computer. CaF_2 windows (3-mm thick) with a 0.5-mm path length were used for the solution spectrum. The crystal spectrum was obtained by transferring HbACO crystals in crystallization buffer to a CaF_2 window (3-mm thick) under a CO atmosphere and overlaying a second CaF_2 window without noticeable air pockets. A total of nine spectra were collected at 0.1-cm^{-1} data intervals, averaged, and corrected for zero base-line absorbance at 1990 and 1925 cm^{-1}. The spectral deconvolution parameters are shown in Table I. The residual difference between the observed spectrum and the sum of the bands of deconvolution is shown at the base line for each spectrum.

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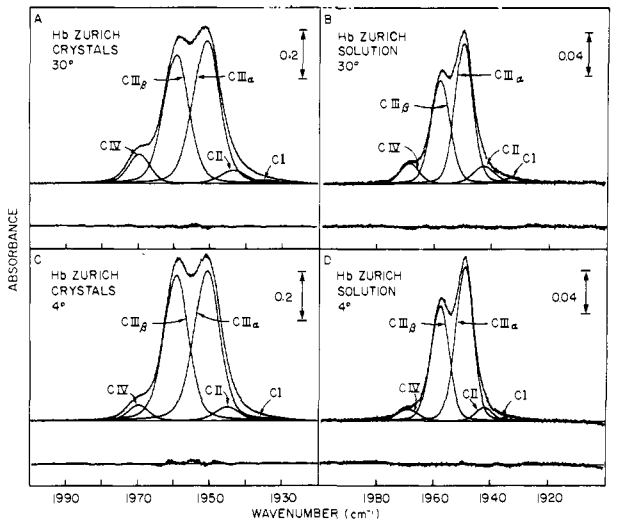

Figure 2. Infrared spectra of HbZhCO in crystals and in solution: (A) crystals at 30 °C, (B) solution at 30 °C, (C) crystals at 4 °C, (D) solution at 4 °C. Conditions and spectral methods are equivalent to those described for HbA in Figure 1. The residual difference between the observed spectrum and the sum of the bands of deconvolution is shown offset below the base line for each spectrum.

band near 1951 cm^{-1} under normal solution conditions, the human mutant Hb Sydney CO (β67 Val→Ala) exhibits a β CIII band at 1955 cm^{-1} and an intensified CIV band at 1968 cm^{-1}.[10] Thus, replacement of Val by the less bulky Ala at Ell, a residue in van der Waal's contact with the CO ligand, results in changes in solution spectra not unlike those found for HbACO upon crystallization.

The β E7 distal His to Arg substitution in HbZhCO (Figure 2) also results in marked changes in the C-O stretch bands, i.e., in solution the β CIII band is at 7-cm^{-1} higher frequency and band CIV is enhanced. The spectra for the normal α subunits of HbZhCO are similar to α HbACO spectra. In solution, the α CIII band of HbZhCO is more intense than the β CIII band. Upon crystallization, the two CIII bands become closer in intensity (Table I). Decreasing the temperature of either crystals or solutions from 30 to 4 °C shifts intensity from minor conformers (CI, CII, CIV) to the major conformer (CIII); thus, interconversion among conformers occurs in crystals as well as solutions. With HbZhCO, crystallization has little effect upon ν_{CO} values. Therefore ligand binding site structures remain essentially unchanged.

In summary, C–O stretch bands provide direct evidence of differences and similarities between crystalline and solution states in terms of conformer structures and relative conformer stabilities. The ν_{CO} values reflect a marked change in ligand binding site structure for the β subunit of HbACO upon crystallization but very little change in the α subunit. With HbZhCO, crystal formation has a smaller effect upon conformer structures. With both hemoglobins, crystallization alters the relative stability of conformers. These infrared results provide evidence that discrete, interconvertible conformers of roughly comparable structures are present in both crystals and solutions. It is also clear that ligand infrared spectra can be uniquely useful for the comparison of crystal and solution protein structures at ligand binding sites.

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Catalysis of *N*-Alkyl-1,4-dihydronicotinamide Oxidation by a Flavopapain: Rapid Reaction in All Catalytic Steps

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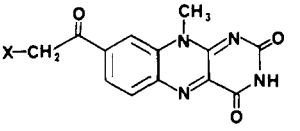
In the course of research on the design and construction of new enzymes we have succeeded in linking various flavins to papain, converting this hydrolytic enzyme into semisynthetic enzymes capable of catalyzing the oxidation of *N*-alkyl-1,4-dihydronicotinamides with high efficiency.[1-5] The most effective of the flavopapain catalysts prepared to date has been the semisynthetic enzyme obtained by the alkylation of the active site Cys-25 residue of papain with 8α-(bromoacetyl)-10-methylisoalloxazine.[4,5] The k_{cat}/K_m value measured at 25 °C and pH 7.5 under aerobic conditions for the oxidation of *N*-hexyl-1,4-dihydronicotinamide by various electron acceptors as catalyzed by this flavopapain **I**



1; X=papain–S

2; X=H

is in the vicinity of 10^6 M^{-1} s^{-1}. This constitutes an approximately 10^3-fold rate increase when compared to the second-order rate constant for the corresponding oxidation reaction catalyzed by the model compound **2**. To understand better the origin of the efficiency of this flavopapain it is important to dissect the observed rate parameters measured under turnover conditions into the microscopic rate parameters for the reaction. Through elucidation of the way in which this rate acceleration arises, it may be possible to learn how to optimize the design of other semisynthetic enzymes. Furthermore, the placement of cofactors into known three-dimensional environments permits the systematic investigation of the factors important for efficient catalysis.

We have used anaerobic conditions and stopped-flow techniques[6] to investigate the individual steps in the oxidation of *N*-alkyl-1,4-dihydronicotinamides catalyzed by flavopapain. We found that at 25.0 °C, under conditions of substrate in excess, the reduction of flavopapain by the *N*-alkyl-1,4-dihydronicotinamides

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