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DIRECT MEASUREMENT OF CARBON MONOXIDE BOUND TO DIFFERENT SUBUNITS OF HEMOGLOBIN A IN SOLUTION AND IN RED CELLS BY INFRARED SPECTROSCOPY

William T. Potter, Jo H. Hazzard, Shosuke Kawanishi[†], and Winslow S. Caughey*

Department of Biochemistry, Colorado State University
Fort Collins, CO. 80523

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Summary. Infrared spectra for carbon monoxide bound to α and β subunits of human hemoglobin A have subunit differences near 1950 cm^{-1} and indicate that 92% of the α subunits exist in one conformer and 5% in a second conformer under conditions where 99% of the β subunit is in only one conformation. The sum of the separated subunit spectra is equivalent to the $\alpha_2\beta_2$ tetramer spectrum. CO infrared spectra indicate that CO displaces O_2 from HbO_2 in red cells or in solution preferentially at the β subunits. The measurement of C-O stretch bands provides a direct method for characterization of ligand binding sites within intact cells.

Introduction. Human hemoglobin A (HbA) is a tetrameric protein composed of two α and two β subunits. When isolated, the subunits exhibit different behavior from that of the $\alpha_2\beta_2$ tetramer, e.g., a lack of cooperativity upon oxygen binding and enhanced ligand affinities (1,2). The proper functioning of the HbA tetramer depends upon interactions between the subunits and their intrinsic differences in ligand affinity (3,4). The direct measurement of ligand binding at the different subunits within the intact Hb tetramer is therefore critical for studies of cooperative ligand binding and release. Such measurements can be achieved with infrared spectroscopy which has proven useful for the discrimination between CO bound to different subunits in human Hb mutants where marked changes in heme environment result in distinctly different spectral bands for CO bound to α and β subunits (5). Subtle differences between normal α and β subunits of HbA were also suggested by these spectra. Here we report confirmation of significant differences in the C-O stretch bands in the separated subunits of HbA. These differences are retained in the $\alpha_2\beta_2$ tetramer and provide an effective means for probing differences

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[†] Present address: Department of Public Health, Kyoto University, Kyoto, Japan.
*To whom correspondence may be addressed.

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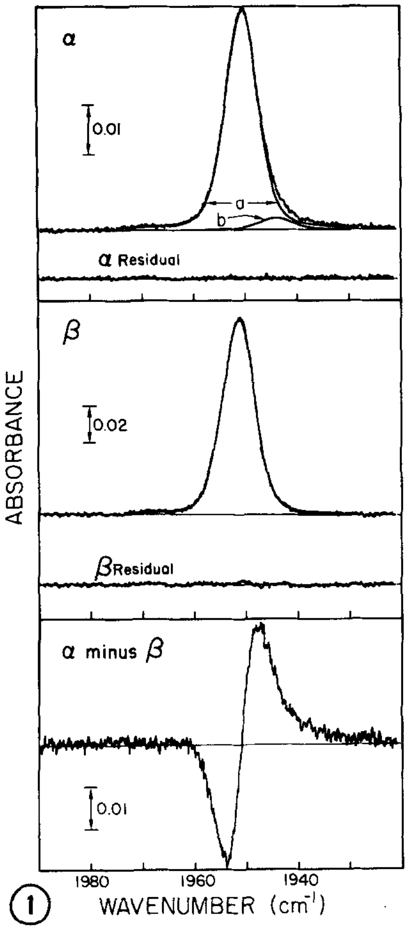
in subunit affinities under physiological conditions. In red cells the replacement of O_2 by CO is shown to occur preferentially at the β subunits.

Materials and Methods. Human HbA hemolysates were prepared from freshly drawn blood (6) and deionized to remove organic phosphates (7). Separated α and β subunits were prepared from HbCO (8) by reaction with p-chloromercuribenzoate (9), followed by ion-exchange chromatography (10,11). After regeneration of free sulfhydryl groups (11), the solutions were concentrated to 10 mM in heme and stored under CO at 4°C (α subunits at pH 8.2 in 0.025 M K phosphate, 0.04 M Tris buffer and β subunits at pH 7.2 in 0.01 M K phosphate buffer) for not more than one week.

Infrared spectra were recorded in cells with CaF_2 windows at 4 ± 0.2°C as described previously (12). Hb concentrations were determined from visible spectra (13). Analysis of infrared spectra by spectrum deconvolution with Gaussian/Lorentzian theoretical curves was performed as previously described (12). ^{13}C NMR spectra for bound $^{13}C^{16}O$ were recorded on a JEOL FX-100 Fourier transform NMR spectrophotometer operating at 25.05 MHz and 19°C (12).

Results and Discussion. The major band for CO bound to β subunit hemes absorbs at a slightly higher frequency than does the CO of the α subunits (Figure 1). This difference in frequency, 1.1 cm^{-1}, is illustrated by the difference spectrum. The α and β subunit spectra also differ in the number of bands contributing to the spectrum. Upon deconvolution the major β subunit absorption is adequately described with a single theoretical curve at 1951.6 cm^{-1} whereas the α subunit spectrum requires two bands, at 1950.5 and 1944.0 cm^{-1} (Figure 1, Table I). Summation of the spectra of the separate subunits gives a reconstructed spectrum for the $\alpha_2\beta_2$ tetramer that is essentially the same as the measured spectrum of HbCO (Figure 2). The close similarity between reconstructed and actual spectra is demonstrated by the very flat residual obtained when the reconstructed spectrum is subtracted from the observed HbA spectrum (Figure 2). These findings indicate that the CO ligand environment differs in the two isolated subunits and that the structures of the fully liganded binding sites in these subunits are not significantly altered upon $\alpha_2\beta_2$ tetramer formation.

The sensitivity of C-O stretch spectra to changes in the protein environment at the heme pocket has been demonstrated for several hemeprotein carbonyls (5). Multiple bands can occur for a single protein subunit. Recently myoglobin carbonyl spectra were shown to consist of four bands whose relative intensities change with changes in temperature, pH and amino acid sequence (12,14,15). The four C-O stretch bands can be ascribed to the presence of



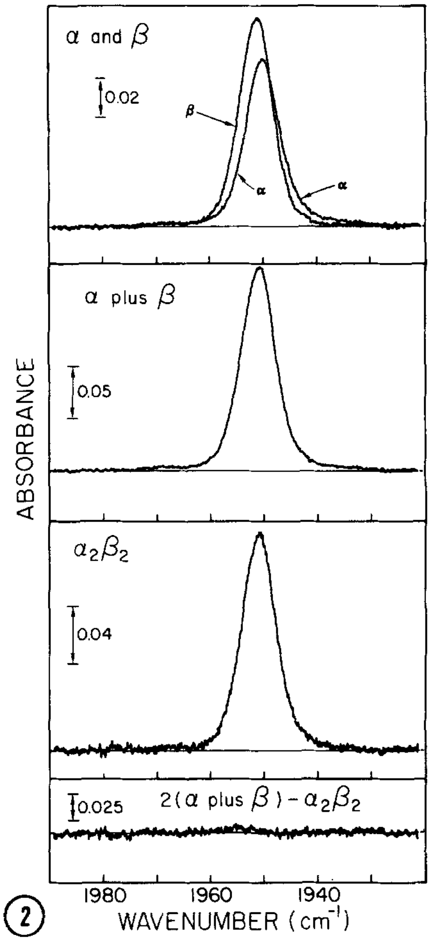


Figure 1. Infrared spectra of CO bound to separated α and β subunits of human HbA at 4°C. Within each figure are the theoretical curves used for deconvoluting each spectrum. The residuals in each case represent the difference obtained from subtraction of theoretical curves from the observed spectrum. The α subunit spectrum requires two bands (a and b) whereas the β subunit spectrum requires only one band for deconvolution. Theoretical band shapes are 65% Gaussian, 35% Lorentzian character for α subunits and 75% Gaussian, 25% Lorentzian character for β subunits. Band parameters are listed in Table I. The difference spectrum (α-β) shows the subtraction of the β subunit spectrum from the α subunit spectrum. The α and β subunit spectra were normalized to the same total intensity prior to subtraction.

Figure 2. Comparison of infrared spectra of CO bound to separated subunits with the spectrum for intact HbACO. Top: Superimposed spectra of the separated subunits of Figure 1. Upper middle: A "reconstructed" Hb tetramer spectrum obtained by adding the α and β subunit spectra. Lower middle: Spectrum of HbA in 0.025 M K phosphate, 0.04 M Tris buffer at pH 8.2. Bottom: Difference spectrum obtained by subtracting the HbA spectrum from the "reconstructed" tetramer spectrum. The spectra were normalized to the same total intensity prior to subtraction.

four discrete, rapidly interconverting conformers. Infrared evidence for rapidly interconverting conformers has also been reported for human Hbs A and Zurich [hisE7(63)β→arg] (16); however, a thorough analysis of HbACO spectra is

Table I. Infrared and ^{13}C NMR Parameters for CO Bound to Separated Subunits of HbA at 4°C

	ν_{CO} (cm^{-1})	$\Delta\nu_{1/2}$ (cm^{-1})	%A[a]	δ[b] (ppm from TMS)
α	1944.0 ± 0.1	7.5 ± 0.1	5.0	206.71 ± 0.05
	1950.5	7.2	92.4	
β	1951.6	7.2	99.0	206.06

[a] Percent of total integrated intensity. For the α-SH subunit, 2.6% of the total area was observed in bands of low intensity at 1969 and 1935 cm^{-1}. For the β-SH subunit, 1% of the total area was observed in a minor band at 1969 cm^{-1}. [b] Only one resonance was observed for each subunit. The difference in chemical shift under these conditions, 0.65 ppm, is comparable to that observed in the Hb tetramer, 0.56 ppm (19).

complicated by the presence of two types of very similar subunits. Evidence from X-ray crystallography suggests the α and β subunits of HbACO have only minor structural differences at the heme ligand binding sites (17) but distinct structural differences can be observed by ^{13}C NMR (18,19) and by infrared spectroscopy (Table I and Figures 1 and 2). The much faster time scale of the infrared experiment compared to either X-ray crystallography or NMR spectroscopy allows the ready detection of C-O stretch bands for two structural conformers of CO bound to the α subunits whereas the β subunits exist almost exclusively as a single CO structure. The significance of the two very minor bands in the spectra presented here (Table I) is currently being investigated. Four C-O stretch bands are evidently a common feature of Hb and Mb carbonyls (20).

The ability to distinguish between ligands at α and β subunits with infrared spectroscopy provides a direct and clear means for the determination of differences in subunit reactivities even within the intact red cells. Less direct methods have resulted in contradictory interpretations of relative affinities of subunits in the HbA tetramer (21). The replacement of O_2 ligands by CO in human packed red cells can be clearly observed to occur preferentially at the β subunits when fully oxygenated cells are titrated with CO (Figure 3). The band maximum position shifts 0.6 cm^{-1} to lower frequency in the

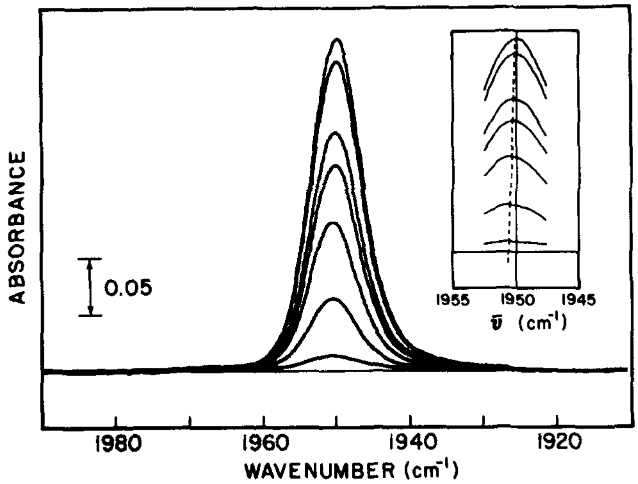


Figure 3. Infrared spectra obtained during the titration of HbO_2 with CO in packed human red cells at 4°. Spectra were recorded at 5,21,43,61,72,93 and 100% CO saturation. The remaining heme sites were liganded with O_2. The insert shows the position of the maximum absorbance on an expanded X-axis. A continuing shift of the absorbance maximum to lower frequencies can be noted as the degree of saturation with CO is increased.

course of the CO titration. The difference in band position between partially CO-liganded and fully liganded spectra are illustrated by the difference spectra in Figure 4. At very low CO levels, only the β subunit is liganded with CO. As more CO is added, the α subunits also bind CO; the band maximum shifts to lower frequency and the difference between the maximum and minimum absorbances in the difference spectra decreases, as plotted in Figure 4. A similar preference of CO for β subunits is observed with purified human HbA solutions; when 30% of the O_2 has been displaced by CO, the frequency of the C-O band maximum is located 0.5 cm^{-1} higher than the frequency observed for the tetramer completely liganded with CO. In contrast, our preliminary infrared experiments on the titration of deoxyHbA in packed red cells with CO indicate a slight preference for CO binding at the α subunits. These deoxyHb findings are in accord with proton NMR studies on CO binding to isolated deoxyHbA (22) and with CO affinities of Fe-Mn hybrid Hbs (23). Our infrared evidence for initial replacement of O_2 by CO at the β subunits is consistent with the relative affinities between O_2 and CO for the separated subunits determined by visible spectroscopy (24) but does not support the recently reported implica-

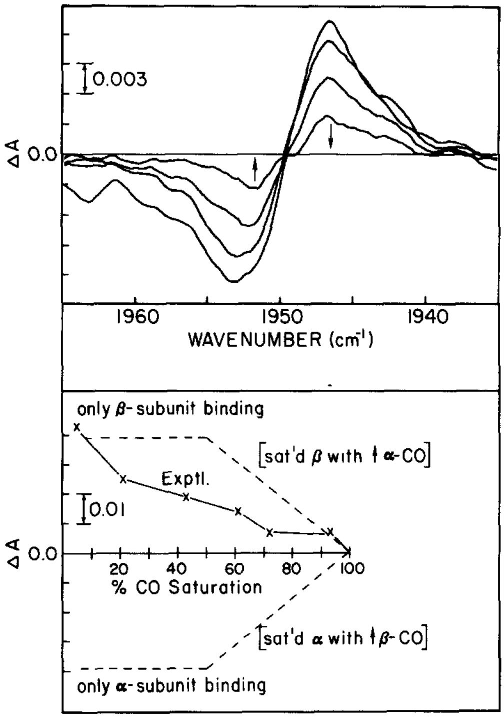


Figure 4. Analysis of infrared spectra of Figure 3. Top: Difference spectra obtained by subtracting spectra at partial CO saturations from the spectrum of fully saturated HbACO in packed human red cells. All spectra were normalized to the same intensity prior to subtraction. Difference spectra for 21,43,61 and 72% CO saturations are shown. Absorbances above 1950 cm^{-1} become less negative and those below 1950 cm^{-1} become less positive with increasing CO saturation (indicated by the arrows). Bottom: Plot of the total difference between the minimum and maximum absorbances of each difference spectrum vs. the percent CO saturation. Theoretical curves (---) show results expected if one subunit type (α or β) were to become completely saturated with CO before the other subunit begins to bind CO. The experimental curve (-x-) indicates the CO replacement of O_2 is preferentially at the β subunits but some binding to the α subunit occurs prior to the complete replacement of O_2 at the β subunits.

tion from ultraviolet spectral studies that the O_2/CO partition constant is the same for both α and β subunits in the fully liganded HbA tetramer (25).

The infrared methodology presented here can be expected to have widespread utility in the direct determination of relative subunit reactivities in Hbs under physiological conditions.

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