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Effect of Differences in Optical Properties of Intermediate Oxygenated Species of Hemoglobin A_0 on Adair Constant Determination†

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ABSTRACT: Careful evaluation of the so-called isosbestic properties of oxygenated and deoxygenated hemoglobin spectra demonstrates that the spectral changes are not strictly linear with respect to the degree of saturation. In order to quantify the extent of nonlinearity, optical measurements of O_2 binding to human hemoglobin were made at different wavelengths in the Soret region approaching the presumed isosbestic point. The results indicate that the extinction coefficient of intermediate oxygenated hemoglobin is 1% less than that of the fully oxygenated hemoglobin, with a resulting 3% (±0.15%) nonlinearity effect on measurements taken at the peak of the oxygenated hemoglobin spectrum (414 nm). The lack of isosbestic conditions allows one to investigate the functional properties of the oxygenated intermediates directly. The small difference in the absorbance of different oxygenated species has practically no influence on the determination of Adair constants at wavelengths removed from the critical isosbestic region.

Spectrophotometric techniques have been used extensively over the past few decades in the analysis of ligand binding to hemoglobin and are a major source of information about the functional properties of the molecule. A basic assumption of these techniques is the linear relationship between O_2 binding and the concomitant optical density changes. Each intermediate ligated species is assumed to have spectral properties based simply upon a weighted contribution of filled and empty binding sites. A key question, pointed out long ago by Gibson (1959), concerns the validity of this assumption.

Analysis of the wavelength dependence of spectrally determined O_2 binding constants of hemoglobin (Hb)[1] (Enoki & Tyuma, 1964; Mills et al., 1976; Imaizumi et al., 1978; Imai, 1982; Gill et al., 1987) has shown that within experimental error no significant alteration in the constants is observed, suggesting the validity of the linearity between spectral changes and degree of saturation. On the other hand, early studies on horse Hb found a discrepancy between spectral measurements of the O_2 binding curve and direct measurements made by gasometric techniques (Rifkind & Lumry, 1967). Possible differences in the optical properties of the two types of Hb chains have also been suggested (Nasuda-Kouyama et al., 1983). Furthermore, recent work on the kinetics of CO binding to human Hb required at least three spectrally distinguishable species for deconvolution of the data (Hofrichter et al., 1985).

Since interpretation of the cooperative properties of hemoglobin relies to a large extent on O_2 binding curves gained by precision spectral data, we wish to quantify the possible optical nonlinearity for O_2 binding. In this study, we determine the degree of optical nonlinearity for O_2 binding to human HbA_0 and its effect on resolution of the Adair constants. Interest in such analysis also comes from the possibility of investigating the binding properties of the oxygenated intermediates directly if the ligated species have different optical properties.

Experimental Procedures

Solution Conditions. Hb samples, isolated by the standard procedures of Williams and Tsay (1973) and stored in deionized water, were reduced for 5 h at 4 °C with the enzymic reducing system of Hayashi et al. (1973). The final solution conditions of 2 mM heme, 20 mM IHP, 0.1 M HEPES, 0.1 M NaCl, and 1 mM Na_2EDTA, 25 °C, pH 7.0, were obtained by mixing the appropriate buffer solution with Hb in deionized water. These conditions were chosen for two reasons: (1) The entire O_2 binding curve can be measured rapidly, largely due to the negative heterotropic effect of IHP. (2) These conditions have been found in a recent study (Di Cera et al., 1987a) to result in a highly stable environment for HbA_0, minimizing both metHb formation (less than 1% during the time frame of an O_2 binding curve) and possible contamination effects. The IHP was used in excess saturating amounts to avoid possible complications in the spectral measurements due to release of this effector during oxygenation, even though such effects are minimal (Imaizumi et al., 1978).

Differential O_2 Binding Curve Measurements. Oxygen binding curves were obtained spectrophotometrically by a

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[1] Abbreviations: Hb, hemoglobin; metHb, methemoglobin; oxyHb, oxygenated hemoglobin; deoxyHb, deoxygenated hemoglobin; HEPES, *N*-(2-hydroxyethyl)piperazine-*N'*-2-ethanesulfonic acid; EDTA, ethylenediaminetetraacetic acid; IHP, inositol hexaphosphate.

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differential technique where changes in O_2 fractional saturation are measured upon precise logarithmic changes in the O_2 partial pressure (Dolman & Gill, 1978; Gill et al., 1987). The sample is held vertically as a thin layer between a gas-permeable membrane and a glass window in a gas-tight cell. The technique allows verification of equilibration of the sample with the gaseous ligands as an unchanging optical signal. A Cary 219 spectrophotometer was used for the optical density measurements.

The data were taken as changes in optical density upon stepwise dilution of the O_2 partial pressure, as described in detail elsewhere (Gill et al., 1987). The general fitting equation for spectrally determined binding data is

$$\Delta OD = \Delta OD_T \theta \quad (1)$$

where ΔOD, the optical density change observed when the degree of saturation (θ) changes from zero to θ, is a linear fraction of the total optical density change (ΔOD_T) observed in saturating the molecule ($\theta = 1$).[2] The degree of saturation θ is given by

$$\theta = \frac{1}{4} \frac{\beta_1 x + 2\beta_2 x^2 + 3\beta_3 x^3 + 4\beta_4 x^4}{1 + \beta_1 x + \beta_2 x^2 + \beta_3 x^3 + \beta_4 x^4} \quad (2)$$

where x is the O_2 activity and β_i is the overall Adair constant (Adair, 1925) for the reaction $Hb + iO_2 \rightleftharpoons Hb(O_2)_i$. Equation 1 assumes that only two optically distinct species exist and therefore that the optical density changes are linearly related to the degree of saturation θ. The existence of optical nonlinearity during ligation requires modification of eq 1 (see Results).

Fitting parameters were estimated by least-squares minimization, using the Gauss–Newton method as modified by Marquardt and others (Frazier & Suzuki, 1973). One standard deviation confidence intervals on critical parameters were determined by F tests (Magar, 1972). A Hewlett Packard 9000/300 computer was used for all the data analysis.

RESULTS

Change of the Isosbestic Point upon Oxygenation. Assumption of linearity between optical density changes and oxygenation of human Hb implies the presence of only two spectrally distinguishable species, corresponding to the ligated and unligated subunits. Therefore, the α and β chains, as well as all possible quaternary states, are assumed to have identical optical properties. If so, a single isosbestic point should be observed between the deoxygenated state and all intermediate stages of oxygenation.

Figure 1 shows the spectra of the fully oxy, deoxy, and partially oxygenated (10%) hemoglobin states in the isosbestic region from 420 to 423 nm. The data were obtained by means of the thin-layer cell (Dolman & Gill, 1978). The experiment was carried out at 12 °C where the normal base-line drift due to oxidation was negligible, and, moreover, the entire experiment was performed within 5 min, essentially eliminating any concern of metHb formation. The observed change in the isosbestic point upon oxygenation demonstrates the absence of linearity between optical changes and degree of ligation. This observation was also made in the visible region, at three presumed isosbestic points (549, 569, and 586 nm), on a sample of stripped Hb (HbA_0 in deionized H_2O), thus excluding any complications due to IHP or chloride anions which are known to alter the spectrum of oxyHb (Enoki & Tyuma, 1964; Adams & Schuster, 1974; Imaizumi et al., 1978).

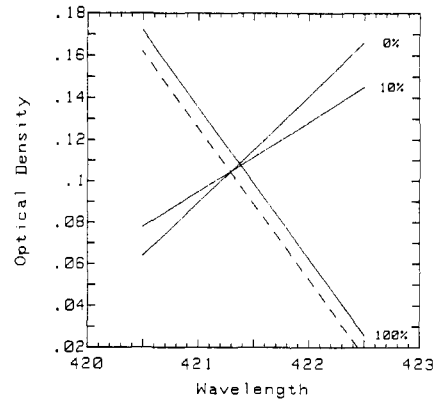

FIGURE 1: Demonstration of the shift in the isosbestic point for HbA in the region of 421.5 nm at various degrees of saturation (indicated in the figure). Experimental conditions were 2 mM heme, 0.1 M HEPES, 0.1 M NaCl, 1 mM Na_2EDTA, and 20 mM IHP, pH 7.0, 12 °C, with the enzymic reducing system of Hayashi et al. (1973). The layer thickness was 0.0025 cm. The dashed line depicts the presumed spectrum of the oxygenated intermediates (see text).

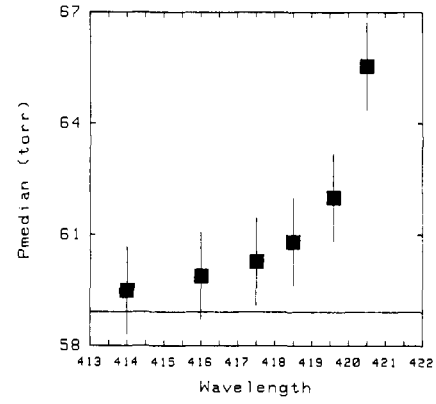

FIGURE 2: Wavelength dependence of the median partial pressure, p_m, for O_2 binding to human HbA_0. Experimental conditions were 2 mM heme, 0.1 M HEPES, 0.1 M NaCl, 1 mM Na_2EDTA, and 20 mM IHP, pH 7.0, 25 °C, with the enzymic reducing system of Hayashi et al. (1973). The layer thickness was 0.0025 cm. The data span the wavelength region from 414 to 420.5 nm. The plotted values were determined by fitting the data to eq 1, and the vertical bars represent twice the standard errors. The horizontal line indicates the value of p_m corrected for nonlinearity (see legend to Figure 3).

The slit-width dependence of the spectra in Figure 1 was also studied, and slit-width values greater than 2.0 nm were found to distort the spectra. Thus, a slit width of 0.6 nm was used, the smallest obtainable under these conditions.

An estimate of the nonlinearity effect can be made from Figure 1. Assuming parallel spectra (see below) for the fully oxygenated and partially oxygenated states (dashed line), the difference in absolute absorbance between the two spectra is about 0.009 optical density unit, or about 1% of the total optical density at the oxygenated peak (414 nm).

Wavelength Dependence of the O_2 Binding Curve. A quantitative estimation of the nonlinearity effect is best made from simultaneous fitting of O_2 binding curves obtained at various wavelengths approaching the isosbestic region. As one approaches the isosbestic region, the nonlinear effect takes on a relatively larger proportion of the total optical density change, and the shape of the binding curve is thus altered. Binding curves were measured at six different wavelengths (from 414 to 420.5 nm) and analyzed in terms of eq 1 assuming linearity between optical changes and degree of ligation. As shown in

[2] Extending eq 1 to the case where θ changes from θ_{i-1} to θ_i leads to $\Delta OD_i = \Delta OD_T(\theta_i - \theta_{i-1})$, which is the fitting equation for data taken by means of the thin-layer apparatus. In this case, ΔOD_i is the optical density change observed in going from θ_{i-1} to θ_i, upon a stepwise dilution of the ligand partial pressure (Gill et al., 1987).

Figure 2, there is a drastic wavelength dependence of the p_{median}. Similar observations have been reported in the visible region (Nasuda-Kouyama et al., 1983).

The data were then analyzed according to a modified form of eq 1, in order to quantify the extent of nonlinearity. In general terms, the optical density change ΔOD in eq 1 can be considered to arise from weighted contributions of the different ligated species, so that

$$\Delta OD = \frac{\Delta OD_{T1}\beta_1 x + 2\Delta OD_{T2}\beta_2 x^2 + 3\Delta OD_{T3}\beta_3 x^3 + 4\Delta OD_{T4}\beta_4 x^4}{4[1 + \beta_1 x + \beta_2 x^2 + \beta_3 x^3 + \beta_4 x^4]} \quad (3)$$

Each ΔOD_{Ti} term in eq 3 represents the total optical density change due to oxygenation of a subunit in the ith ligated species. When the optical density change is the same for all ligated species, then $\Delta OD_{T1} = \Delta OD_{T2} = \Delta OD_{T3} = \Delta OD_{T4}$, and eq 3 collapses into eq 1. In such a case, perfect linearity would be obtained between optical changes and degree of saturation θ.

In the absence of linearity, a difference in the optical properties of different ligated species must exist. Analysis of the binding data according to eq 3 yielded nearly identical values for ΔOD_{T1}, ΔOD_{T2}, and ΔOD_{T3} (although ΔOD_{T3} was poorly determined due to the negligible best-fit value for β_3; see Discussion), indicating that optical differences exist largely between the fully and partially oxygenated species, so that

$$\Delta OD = \frac{1}{4}\frac{\Delta OD_{Ti}\beta_1 x + 2\Delta OD_{Ti}\beta_2 x^2 + 3\Delta OD_{Ti}\beta_3 x^3 + 4\Delta OD_{T4}\beta_4 x^4}{1 + \beta_1 x + \beta_2 x^2 + \beta_3 x^3 + \beta_4 x^4} \quad (4)$$

where ΔOD_{Ti} is the optical density change of the oxygenated intermediates, being equal for the singly, doubly, and triply ligated species.

According to Figure 1, it is clear that the extinction coefficient of the intermediates is smaller than that of the fully ligated species; i.e., $\Delta OD_{Ti} < \Delta OD_{T4}$. Therefore, one can express ΔOD_{Ti} as $\Delta OD_{T4} - \delta$, where δ is a correction factor for nonlinearity, equal to the difference between the spectra of the fully and partially ligated species (see Figure 1). Equation 4 then becomes

$$\Delta OD = \frac{1}{4}[(\Delta OD_T - \delta)\beta_1 x + 2(\Delta OD_T - \delta)\beta_2 x^2 + 3(\Delta OD_T - \delta)\beta_3 x^3 + 4\Delta OD_T\beta_4 x^4]/[1 + \beta_1 x + \beta_2 x^2 + \beta_3 x^3 + \beta_4 x^4] \quad (5)$$

where ΔOD_{T4} has been rewritten as ΔOD_T for simplicity. Upon rearrangement, we obtain

$$\Delta OD = \Delta OD_T\theta - \delta\frac{1}{4}\frac{\beta_1 x + 2\beta_2 x^2 + 3\beta_3 x^3}{1 + \beta_1 x + \beta_2 x^2 + \beta_3 x^3 + \beta_4 x^4} \quad (6)$$

which gives eq 1 corrected for nonlinearity.

The six binding curves measured from 414 to 420.5 nm were simultaneously fitted to eq 6. The correction factor δ was assumed to be constant over the wavelength range studied.[3] Since the actual Adair constants are wavelength independent, the fitting procedure is highly constrained, yielding a precise value for δ. The result of the simultaneous analysis is shown in Figure 3. The effect of nonlinearity, expressed by the ratio $\delta/\Delta OD_T$ at 414 nm, is determined as 3% ± 0.15%.

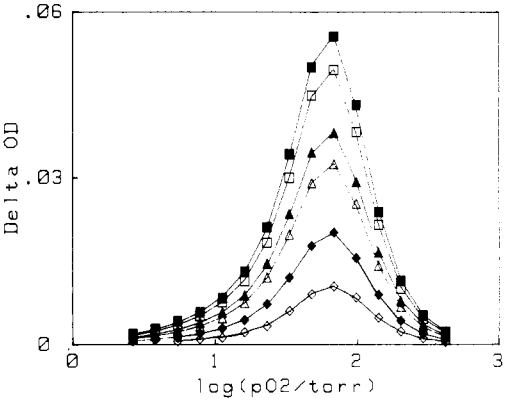

FIGURE 3: Differential O_2 binding curves taken separately over the wavelength range 414 to 420.5 nm: 414 nm (■); 416 nm (□); 417.5 nm (▲); 418.5 nm (△); 419.6 nm (♦); 420.5 nm (◇). Experimental conditions are given in the legend to Figure 2. The data were simultaneously fitted to eq 6 in the text to determine the correction factor δ for optical nonlinearity. The Adair constants, as calculated from the simultaneous fit, are $\beta_1 = 0.021$ (±0.001) torr^{-1}, $\beta_2 = 0.00035$ (±0.00004) torr^{-2}, $\beta_3 = 0$ (±6.6 × 10^{-10}) torr^{-3}, $\beta_4 = 8.3 \times 10^{-8}$ (±3.0 × 10^{-9}) torr^{-4}. The values of ΔOD_T at each wavelength are ΔOD_T(414.0) = 0.2886 (±0.0005), ΔOD_T(416.0) = 0.2565 (±0.0005), ΔOD_T(417.5) = 0.1976 (±0.0004), ΔOD_T(418.5) = 0.1676 (±0.0004), ΔOD_T(419.6) = 0.1027 (±0.0003), ΔOD_T(420.5) = 0.0525 (±0.0002). The value of the correction factor δ is 0.00889 (±0.00044). The standard error of a point of the simultaneous fit is 0.00015 optical density unit.

Analysis of the Oxygenated Intermediates at the Hb-$(O_2)_4$–Hb Isosbestic Point. The differences observed for the optical properties of the partially ligated intermediates relative to the fully ligated HbA_0 can be exploited to investigate the binding properties of the intermediates directly. At the Hb-$(O_2)_4$–Hb isosbestic point, optical density changes due to the fully ligated and deoxy species are absent. Thus, any spectral changes observed during oxygenation are attributable to the intermediate species $Hb(O_2)$, $Hb(O_2)_2$, and $Hb(O_2)_3$. At this isosbestic point, $\Delta OD_T = 0$ (see eq 6), and one can measure the optical correction factor δ of the intermediates directly according to the equation:

$$\Delta OD = -\delta\frac{1}{4}\frac{\beta_1 x + 2\beta_2 x^2 + 3\beta_3 x^3}{1 + \beta_1 x + \beta_2 x^2 + \beta_3 x^3 + \beta_4 x^4} \quad (7)$$

Since the data taken with the thin-layer cell are differential data, they are expected to show both positive and negative optical density changes at this isosbestic point, as predicted by eq 7. Figure 4 illustrates binding data taken at the Hb-$(O_2)_4$–Hb isosbestic point which displays the predicted shape. The correction factor δ was determined as 0.00897 optical density unit, as expected (see Figure 1), and the determined Adair constants are in good agreement with those obtained independently in the simultaneous fit of the binding data taken at different wavelengths (see Figure 3).

Discussion

Influence of Optical Nonlinearity on Adair Constant Determination. The effect of nonlinearity on Adair constant determination has been a major concern in the past few decades (Gibson, 1959; Rifkind & Lumry, 1967; Imaizumi et al., 1978). Yet, precise estimation of this effect has not been reported.

Previous work (Mills et al., 1976; Imaizumi et al., 1978; Gill et al., 1987) has shown that the shape of the O_2 binding curve is, within experimental error, independent of the wavelength

[3] This implies that the spectra of the fully and partially ligated species are parallel in the wavelength range studied (see Figure 1). Statistical analysis of the simultaneous fit by means of the F test has demonstrated the validity of this assumption with a confidence >67%.

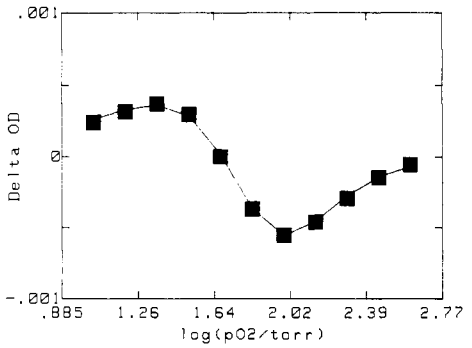

FIGURE 4: Oxygen binding data at the $Hb(O_2)_4$–Hb isosbestic point in the Soret region (421.4 nm). Experimental conditions are given in the legend to Figure 2. The theoretical curve was drawn from eq 7 in the text, with the following best-fit parameters values (the confidence intervals at one standard deviation, determined by F testing, are reported in parentheses): $\beta_1 = 0.027$ (0.020, 0.031) $torr^{-1}$, $\beta_2 = 0.00040$ (0.00032, 0.00041) $torr^{-2}$, $\beta_3 = 0$ (3.5×10^{-8}) $torr^{-3}$, $\beta_4 = 8.2 \times 10^{-8}$ (7.7×10^{-8}, 8.9×10^{-8}) $torr^{-4}$, $\delta = 0.00897$ (0.00875, 0.00912). The standard error of a point is 0.000017 optical density unit.

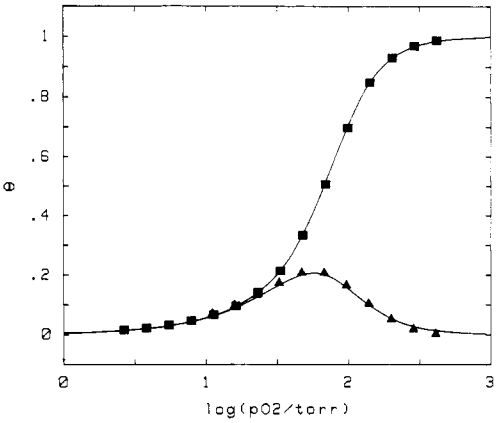

FIGURE 5: Degree of saturation θ versus logarithm of O_2 partial pressure as determined at 414 nm (squares) and at the $Hb(O_2)_4$–Hb isosbestic point (triangles) at 421.4 nm. The data were obtained in differential form (see Figures 3 and 4) and transformed to degree of saturation for comparison. The theoretical lines were drawn from eq 6 and 7 by summation of the stepwise optical density changes at each step. The values of the Adair constants are the same for both curves and are reported in the legend to Figure 3. One can see how the degree of saturation of the intermediates reaches a maximum in the middle region of the binding curve and goes to zero at both ends. Furthermore, the figure demonstrates that the data shown in Figures 3 and 4 resemble the derivative of the binding curve θ.

used, indicating the absence of complications arising from nonlinearity. However, the data were for the most part taken at peaks and troughs, where the effects of optical nonlinearity are minimal. In order to assess the effect of nonlinearity, one should measure the binding curve at various wavelengths while approaching an isosbestic region. As seen from eq 6, the nonlinearity effect makes a progressively larger contribution to the observed signal as ΔOD_T decreases and can therefore be quantified by a simultaneous fitting procedure. For the situation investigated in detail above, the correction factor δ is equal to 0.00889 optical density unit and represents only 1% of the absolute absorbance at 414 nm, or 3% of ΔOD_T at 414 nm.

With a quantitative estimation of the optical nonlinearity, one can establish whether there is a significant influence on the determination of the Adair constants for O_2 binding. Simulation studies were carried out by introducing an extent of nonlinearity from 0 to 10% in the optical properties of the intermediates relative to $Hb(O_2)_4$. The systematic error induced by neglecting the nonlinearity in the fitting equation, i.e., using eq 1 instead of eq 3, was found to be insignificant below the 3% level. The results of the simulation study were confirmed by analysis of real experimental data, with and without correction for nonlinearity. Therefore, the effect of nonlinearity can be neglected for O_2 binding data taken at the peaks and troughs of the oxyHb spectrum. This conclusion substantiates previous observations (Mills et al., 1976; Imaizumi et al., 1978; Gill et al., 1987).

The possibility of metHb formation as an origin of the observed optical nonlinear effect is ruled out for four reasons. (1) The data represented in Figure 1 were obtained in less than 5 min and under solution conditions that greatly reduce metHb formation (Di Cera et al., 1987a). (2) All binding curves in Figure 2 were obtained under an identical time frame (90 min) and solution conditions, the only variable being the detection wavelength. (3) The type of data shown in Figure 4 was also obtained in a kinetic mode by repeatedly completely oxygenating and deoxygenating the sample. The same magnitude and shape of the optical density changes was observed several times on the same sample and in either the oxygenation or the deoxygenation direction. (4) The reported 3% nonlinear effect was obtained independently from each of the three different experimental strategies represented by Figures 1, 3, and 4.

Direct Investigation of the Oxygenated Intermediates. The lack of isosbestic conditions has important implications for exploring the O_2 binding reactions of the intermediate oxygenated states directly. Since all spectral changes observed at the $Hb(O_2)_4$–Hb isosbestic point are due to the O_2 binding reactions of the singly, doubly, and triply ligated species, direct spectral investigation of these intermediates can be made on the intact Hb molecule. This phenomenon is particularly relevant to the study of Hb functional properties, since the highly cooperative nature of the O_2 binding curve leads to very low populations of the intermediates, which are then difficult to resolve. The data shown in Figure 4 are the first direct investigation of the O_2 binding properties of human Hb intermediates obtained with the intact molecule. The internal consistency of the data obtained at the $Hb(O_2)_4$–Hb isosbestic point is demonstrated by the excellent agreement between the Adair constants obtained at this wavelength and those determined in regions where the effect of nonlinearity is negligible. This is best seen in Figure 5, where the data collected at 414 nm and at the $Hb(O_2)_4$–Hb isosbestic point are compared in a plot of θ versus the logarithm of O_2 partial pressure. As expected, the degree of saturation of the intermediates (triangles) reaches a maximum in the middle region of the binding curve and goes to zero at the ends. Both data sets can be fitted to the same Adair constants.

This consistency allows one to draw quantitative conclusions from the analysis of the functional properties of Hb intermediates, such as the neglibible contribution of the triply ligated species to the oxygenation process. Observation of a negligible population of the $Hb(O_2)_3$ species has recently been reported for a wide range of experimental conditions (Gill et al., 1987; Doyle et al., 1987; Di Cera et al., 1987a) and is confirmed independently here. The adair constant β_3 fits to zero values at all wavelengths studied, with and without correction for nonlinearity.

Implications for the Allosteric Mechanism of Hb Oxygenation. The unmeasurable contribution of the triply ligated species to the oxygenation process has been rationalized within the framework of an allosteric model (Di Cera et al., 1987b) which is based on the stereochemical mechanism proposed by Perutz (1970). On the basis of this model, it is tempting to speculate that the origin of nonlinearity between optical

changes and degree of saturation is due to different spectral properties of the two quaternary states of Hb upon oxygenation. This difference may be attributed at a deeper level to different optical properties of the α and β chains in the two possible quaternary T and R states. Deconvolution of the Hb binding properties observed in the critical isosbestic region will help to characterize the functional properties of the T and the R states at intermediate stages of ligation.

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Folding of Homologous Proteins: Conservation of the Folding Mechanism of the α Subunit of Tryptophan Synthase from *Escherichia coli*, *Salmonella typhimurium*, and Five Interspecies Hybrids†

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ABSTRACT: The equilibrium and kinetic properties for the urea-induced unfolding of the α subunit of tryptophan synthase from *Escherichia coli*, *Salmonella typhimurium*, and five interspecies hybrids were compared to determine the role of protein folding in evolution. The parent proteins differ at 40 positions in the sequence of 268 amino acids, and the hybrids differ by up to 15 amino acids from the *Escherichia coli* α subunit. The results show that all the proteins follow the same folding mechanism and are consistent with a previously proposed hypothesis [Hollecker, M., & Creighton, T. E. (1983) *J. Mol. Biol. 168*, 409; Krebs, H., Schmid, F. X., & Jaenicke, R. (1983) *J. Mol. Biol. 169*, 619] that the folding mechanisms are conserved in homologous proteins. Analysis of the kinetic data suggests that the 15 positions at which the parent proteins differ in the amino folding unit, residues 1-188, do *not* play a role in a rate-limiting step in folding that has been previously identified as the association of the amino and carboxyl folding units [Beasty, A. M., Hurle, M. R., Manz, J. T., Stackhouse, T. S., Onuffer, J. J., & Matthews, C. R. (1986) *Biochemistry 25*, 2965]. One or more of the 25 positions at which the parent proteins differ in the carboxyl folding unit, residues 189-268, *do* appear to play a role in this same rate-limiting step.

Comparative studies on homologous proteins have generally focused on the effect of amino acid changes on the structure, stability, and function (Rossman & Argos, 1981; Bajaj & Blundell, 1984). The results highlight individual residues that play key roles in substrate binding and/or catalysis as well as the general principles that relate amino acid sequence to secondary and tertiary structure.

A closely related question concerns the effects of these amino acid replacements on the folding mechanism. Comparison of the refolding kinetics of ribonucleases from a group of ruminants showed that the same mechanism applied for all variants (Krebs et al., 1983; Lang et al., 1986). From similar studies on another group of ribonucleases, Schmid et al. (1986)

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