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CHAIN HETEROGENEITY IN THE METHEMOGLOBIN-AZIDE EQUILIBRIUM*

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SUMMARY

The absorption spectra of solutions of methemoglobin partially saturated with azide were resolved into the best fitting components of two reference spectra (methemoglobin and methemoglobin azide) by a least-squares curve fitting operation. While good fits of sample spectra in terms of reference spectra were obtained as the extreme values of saturation were approached, poor fits were obtained in the middle region of fractional saturation. The distribution of residuals was markedly wavelength dependent, the greatest excursions being obtained at the isoabsorption point in the 0-100% azide difference spectrum of methemoglobin. The results are attributed to chain differences in an uncooperative tetramer.

The use of absorption spectroscopy for determination of fractional saturation of hemoproteins with ligands frequently yields an inconclusive result. For example, Knowles and Gibson (1) have shown that the absorption spectrum of hemoglobin A partially saturated with oxygen was not well described by a weighted combination of the spectra of oxy- and deoxy-hemoglobin. The wavelength dependence of residuals obtained by least-squares analysis was non-random and closely similar to the family of experimentally obtained difference spectra described earlier (2) which shared the common feature of arising by an apparent shift in the position of absorption bands. The finding of spectral species unlike either oxy- or deoxy-hemoglobin raises doubts about the limits of precision inherent in the techniques of absorption spectroscopy used for determination of fractional saturation. The incorporation of additional spectroscopic species into the curve fitting procedure requires a knowledge of ex-

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tinction coefficients as well as mass action expressions for equilibria involving these spectroscopic species. In view of the several origins of these intermediate spectroscopic states, assumptions concerning the mass action laws would not be justified for the hemoglobin-oxygen system.

The methemoglobin-azide equilibrium, on the other hand, permits one simplifying assumption without being reduced to the level of simplicity presented by the myoglobin-oxygen equilibrium. This equilibrium was demonstrated to be free of cooperative interactions (3) and spectral shifts due to conformational transitions are, therefore, excluded from consideration. A model for binding of azide by methemoglobin was adopted which takes account of chain differences and the properties of this system were simulated using computer techniques. Under conditions of chemical equilibrium the variation in absorbance with fractional saturation at the wavelength of the isoabsorption point between methemoglobin and its azide derivative was predicted to vary in a characteristic manner. The azide-dependent absorbance excursions predicted by the model have been experimentally verified.

EXPERIMENTAL PROCEDURES

Materials

Pooled samples of human blood were obtained from the Tompkins County Hospital. Hemoglobin was prepared by hemolysis of red blood cells as described earlier (4). Whale skeletal muscle myoglobin was obtained from Sigma Chemical Company. Methemoglobin and metmyoglobin were prepared by ferricyanide oxidation. A slight excess of potassium ferricyanide was added to each hemoprotein. Each sample was then gel-filtered through a column of Sephadex G-50 equilibrated with 0.050 M tris-hydrochloride, 0.100 M sodium chloride, pH 7.0. The samples were used on the day of preparation.

Spectroscopic Measurements

A series of solutions were prepared by addition of a constant quantity of a methemoglobin solution to volumetric flasks containing different quantities of sodium azide. Sodium azide was added as a 0.050 M solution which was 0.050 M in Tris and 0.100 M in sodium chloride, pH 7.0. The spectrum of each solution was recorded between 380 and 460 mμ at 1.0 mμ intervals using the computer-interfaced spectrophotometer (1). The spectrum of methemoglobin-azide was obtained with a final azide concentration of 0.050 M. The spectra of methemoglobin and its azide derivative were used as reference spectra to resolve the spectrum of all other solutions into methemoglobin and methemoglobin azide components. This was achieved by a least squared fitting procedure carried out with a digital computer. The computed composition of each solution was used to generate a synthetic spectrum and the synthetic spectrum was compared with the observed spectrum by taking the differnece at each wavelength. This difference spectrum, called a residual spectrum, was examined for a non-random distribution of residuals. A control experiment was conducted with metmyoglobin and azide.

RESULTS AND DISCUSSION

The residual spectra for methemoglobin solutions partially saturated with azide were found to be markedly wavelength dependent. The family of residual spectra obtained displayed isoaborption points at 407, 421 and 437 nm. Positive maxima were obtained at 401 and 427 nm and a negative maximum was obtained at 414 nm. A comparison of the residual spectra with the ligand difference spectrum (methemoglobin azide minus methemoglobin) does not reveal any spectral similarity. The negative maximum of the residual spectrum coincides exactly with the isoabsorption point in the 0-100% ligand difference spectrum and the isoabsorption points at 407 and 421 nm in the residual spectra coincide very nearly with the maxima in the ligand difference spectrum. In contrast, significant residuals were not obtained upon analysis of metmyoglobin solutions partially saturated with azide so that the difficulty in fitting the spectra is specific to hemoglobin. These results are presented in Figures 1 and 2.

It has been known for some time that hemoglobin contains two chemically different types of chain, α and β, and azide difference spectra for the ferric-forms have been presented together with an estimate derived from kinetic data that the β-chain has about two times greater affinity for azide than the α-chain (3). It was, therefore, possible to examine the results of Figure 2 with the idea in mind that they might be due to preferential binding of azide to the β-chain. A solution of methemoglobin and sodium azide will contain α- and β-chains and their azide derivatives. The absorbance of such a solution at any wavelength, A_λ, will be the sum of contributions from four species:

$$A^\lambda = a^\lambda_\alpha + a^\lambda_{\alpha N_3^-} + a^\lambda_\beta + a^\lambda_{\beta N_3^-} \qquad \text{(Eq. 1)}$$

where the subscripts denote the species. The partial absorbance values may be calculated using Beer's Law,

$$a^\lambda_i = \varepsilon^\lambda_i\,(i)1 \qquad \text{(Eq. 2)}$$

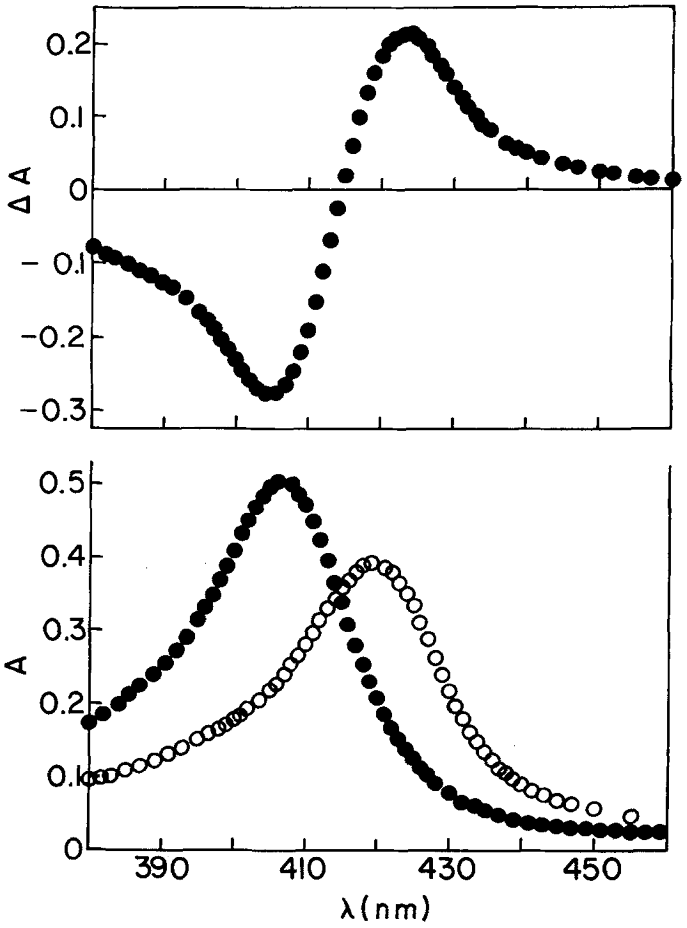

Figure 1. Absorption Spectra of Methemoglobin (●), Methemoglobin Azide (○) and the Azide Difference Spectrum. Absorption spectra were obtained using 0.050 M Tris, 0.100 M NaCl, pH 7.0 at 20° C ± 0.050 M NaN_3.

where a_i^λ is the absorbance of the *i*th species, ε_i^λ is its molar extinction coefficient and (*i*) is its concentration. Analytical expressions for (*i*) were derived from the mass action expression,

$$\alpha + N_3^- \rightleftarrows \alpha N_3^-;$$

$$(\alpha) = C_\alpha/[K_\alpha(N_3^-) + 1] \qquad \text{(Eq. 3)}$$

$$(\alpha N_3^-) = C_\alpha K_\alpha(N_3^-)/[K_\alpha(N_3^-) + 1] \qquad \text{(Eq. 4)}$$

where C_α is the sum of the α-chain forms and K_α is the binding constant. Similar expressions are obtained for the β-chain species. Equation 1 thus becomes:

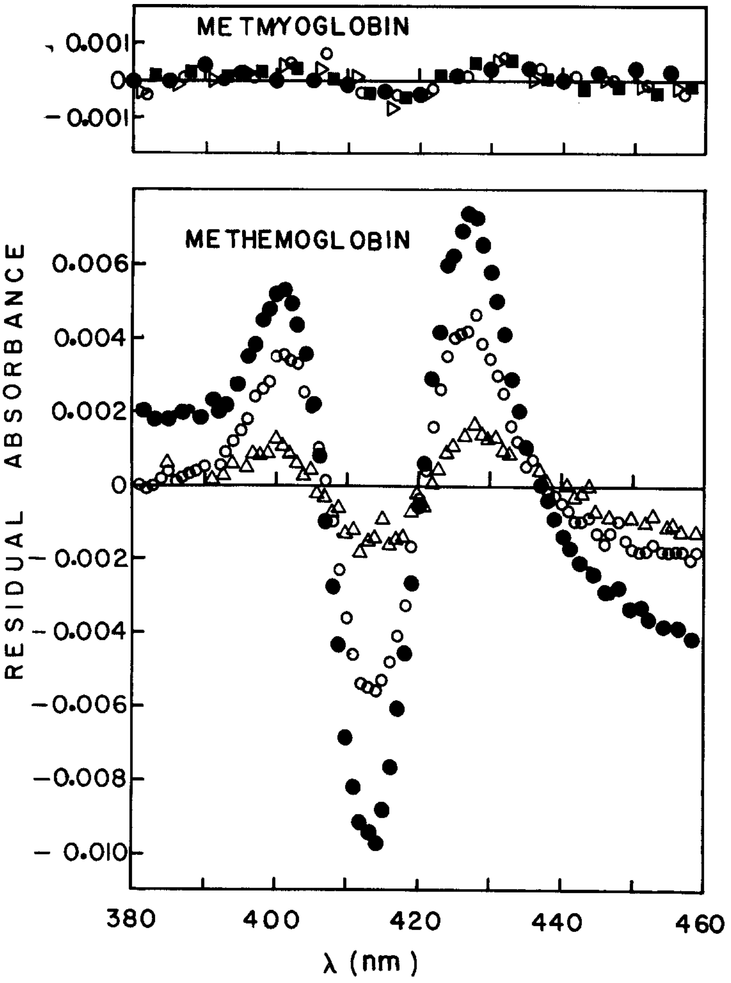

Figure 2. Residual Spectra for the Methemoglobin-Azide and Metmyoglobin-Azide Systems. The residual spectra for the methemoglobin-azide system were obtained at values of fractional saturation equal to 0.11 (△) 0.35 (○) and 0.50 (●). The residual spectra for the metmycglobin azide system were obtained at values of fractional saturation equal to 0.09 (○), 0.41 (●), 0.45 (△) and 0.80 (■).

$$A^{\lambda} = \frac{C_{\alpha} l[\varepsilon_{\alpha}^{\lambda} + \varepsilon_{\alpha N_3^-}^{\lambda} K_{\alpha}(N_3^-)]}{K_{\alpha}(N_3^-) + 1} + \frac{C_{\beta} l[\varepsilon_{\beta}^{\lambda} + \varepsilon_{\beta N_3^-}^{\lambda} K_{\beta}(N_3^-)]}{K_{\beta}(N_3^-) + 1} \qquad \text{(Eq. 5)}$$

The spectroscopic data for the ferric-forms of the α- and β-chains have been presented as azide difference spectra (3) and do not provide directly the information required for simulation of azide-dependent absorbance excursions. This difficulty may be overcome by restricting the analysis of results to the wavelength characterized by the isoabsorption point in the 0-100% azide difference spectrum of methemoglobin, 414.5 nm. The molar extinction coefficient of methemoglobin has a value of 107,000 at this wavelength and, since the azide difference spectra for the isolated chains are offset by about two nanometers, the value of 107,000 must represent an average for the unliganded chains as well as for the azide derivatives. Thus, the case where all values of $\varepsilon_i^{414.5}$ are 107,000 is excluded. Furthermore, the case where both the α- and β-chains display an isoabsorption point at a common wavelength but, with different values of $\varepsilon_i^{414.5}$, can be excluded since a kinetic isoabsorption point cannot be observed at 414.5 nm (3). The azide difference spectra for the isolated chains do, however, provide guidance in assigning the values of $\varepsilon_i^{414.5}$. In view of the two nanometer offset in these difference spectra and the slope of the absorption bands of methemoglobin and methemoglobin azide at 414.5 nm, the values of $\varepsilon_i^{414.5}$ may be expected to vary by as much as 16.5% and 7.5%, respectively, from the mean value of 107,000. With these constraints in mind, the following reasonable values of $\varepsilon_i^{414.5}$ were used in simulating azide-dependent absorbance excursions: α-chain and azide derivative of the β-chain, 97,000; β-chain and azide derivative of the α-chain, 117,000. The association constant for the β-chain, K_β, was treated as a multiple of the association constant for the α-chain, K_α, which was set to 25,000 1/mol in reasonable accordance with literature values (3,5). The concentration of both the α- and β-chains, C_α and C_β, were set to 2×10^{-5} <u>M</u> and the light path, 1, was set to 1.00 mm, these values being similar those used in the experiments described in Figure 2.

The use of these values for simulation of azide-dependent absorbance excursions yields a calculated absorbance of 0.428 at either extreme of saturation of methemoglobin with azide ion. When K_β and K_α are equal, Equation 5 reduces to an identity and no absorbance excursion is predicted. When the value of K_β was taken to be twice that of K_α, a maximum absor-

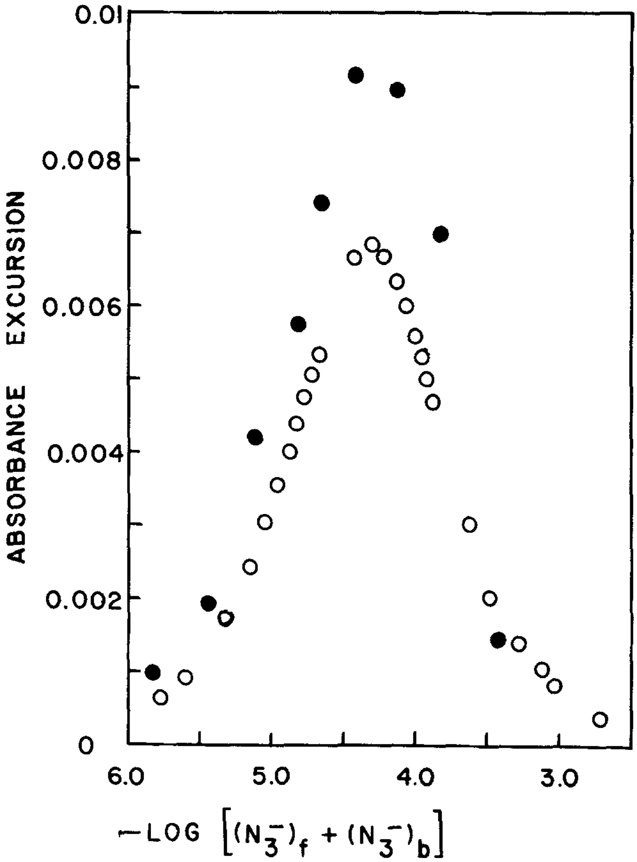

Figure 3. Correlation of Simulated Data for Azide-Dependent Absorbance Excursions and Residual Spectra Amplitudes. Simulated absorbance data (○) was calculated using Equation 5 and subtracted from the abosrbance at the isoabsorption point in the 0-100% azide difference spectrum, 0.428. Experimentally obtained absorbance excursions (●) were obtained as the amplitude of the residual spectra at 414.5 nm.

bance excursion was obtained at 5 x 10^{-5} M azide (free plus bound). The distribution of azide dependent absorbance excursions predicted by Equation 5 closely reproduces the pattern of residual spectrum amplitudes. This correlation between predicted values and experimental results is illustrated in Figure 3. The magnitude of the predicted absorbance excursions depends upon the ratio of K_β to K_α. Other values being constant, an increase of K_β to 4 K_α approximately doubles the magnitude of the excursion with only a slight shift in the position of the maximum to lower total concentrations of azide ion. Larger percentage variations in the values of $\varepsilon_i^{414.5}$ result in directly proportional increases in the predicted excursions with no change in the position of the maximum.

These absorbance excursions require that there be differences in the 0-100% azide difference spectra for the α- and β-chains. Thus, isoabsorption points in the azide difference spectra should be correlated with wavelengths of zero excursion in the residual spectra. Indeed the azide difference spectra reported for the α- and β-chains show isoabsorption points at 406, 421 and 435 nm. These wavelengths correlate with wavelengths of zero excursion in the residual spectra of 406, 421 and 437 nm. Furthermore, the wavelengths where the greatest discrepancies are found between the ligand difference spectra for the α- and β-chains are correlated with the wavelengths of maximum amplitude in the residual spectra. Thus, the entire wavelength dependence of the residual spectra can be correlated with extinction coefficients obtained from the 0-100% azide difference spectra of the individual chains.

Barksdale _et al_. (5) have shown that the relation between fractional spectral change and fractional azide binding is non-linear when followed at one wavelength. They suggested that their result might be due to tetramer-dimer dissociation or to non-heme binding of azide. We have confirmed this result using many wavelengths and offer an alternative explanation of the findings. A preferred order of binding of azide ion to the β-chain of methemoglobin can be manifested under conditions of chemical equilibrium. The methemoglobin-azide equilibrium, therefore, is a four component system and interpolative methods, which presume only two components, will not successfully describe the results of equilibrium binding experiments.

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