ANGULAR DEPENDENCE OF LIGHT SCATTERING LINEWIDTHS FROM HEMOGLOBIN SOLUTIONS

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Received April 16,1974

Summary

We have measured the spectra of light scattered from solutions of oxy and carbon monoxy hemoglobin solutions under a variety of solution conditions, and as a function of scattering angle. In all cases we do not observe the anomalous positive intercepts in the plot of linewidth vs. the square of the scattering vector which have been reported by other workers and attributed to the kinetics of the dimer-tetramer interaction. We believe the anomalies may be attributable to the coincidence of the Helium-Neon wavelength and the 630 nm absorption of methemoglobin.

INTRODUCTION

Inelastic laser light scattering has become a standard technique for the measurement of diffusion coefficients of macromolecules in solution (1,2). If the solution is monodisperse, the light scattering spectrum is expected to be Lorentzian with a half-width at half-height proportional to DK^2, where D is the translational diffusion coefficient of the macromolecule and K is the scattering vector, whose magnitude is given by $(4\pi n/\lambda_0)\sin(\theta/2)$, where n is the index of refraction of the solution, λ_0 is the incident wavelength, and θ is the scattering angle. It is customary when using this technique to measure the half-width at various scattering angles and plot the half-width as a function of K^2. The resulting plot should be a straight line with zero intercept whose slope is a measure of the translational diffusion coefficient of the macromolecule. Deviations from this kind of plot are taken to indicate that the spectrum is not arising solely from the translational diffusion of a monodisperse solution of macromolecules.

Recently Uzgiris and Golibersuch (3) reported an unusual and interesting

anomaly in the light scattering spectra from solutions of hemoglobin. The plots of light scattering linewidth as a function of K^2 all had positive intercepts. These intercepts were interpreted as arising from the hemoglobin dimer-tetramer association-dissociation reaction kinetics, and kinetic rate constants were calculated. Although it had been shown theoretically that the kinetics of macromolecular interaction could conceivably be reflected in the light scattering spectrum (4), there had been no previous experimental evidence for this effect. However, as Uzgiris and Golibersuch pointed out, the details of their observations could not be explained by any existing theory.

We have been measuring light scattering spectra from hemoglobin solutions for some time and have observed none of the anomalies reported. We have recently performed the experiments under the conditions specified by Uzgiris and Golibersuch. In this communication we report the details of our observations. No anomalies are seen, and a possible explanation for their observations has been deduced.

MATERIALS AND METHODS

Human blood was freshly drawn from a normal subject (male, 24 years old) using heparin as an anticoagulant. Cells were washed in .9% saline, pH 7. Hemoglobin was extracted using the standard toluene procedure of Drabkin (5) and purified on a Sephadex G-25 column (6). Samples were dialyzed to the desired solution conditions, were stored as carbon monoxy hemoglobin (HbCO) at 4°C, and were used within two weeks. Oxyhemoglobin (HbO_2) samples were prepared by gently bubbling oxygen through the HbCO solution, which was simultaneously illuminated by an ultraviolet lamp. All HbO_2 samples were used immediately and discarded within 12 hours. For all experiments the optical absorption spectra were measured with a Cary 14 immediately before and after the light scattering spectra were obtained. This assured not only precise determination of the ligand and concentration, but also assured the absence of any significant amounts of methemoglobin.

Solutions were filtered through .22μ Millipore filters which had been twice boiled to remove any surfactants. Solutions were filtered directly into the light scattering cells, which were standard fluorescence cells (Hellma, Inc. Models No.

110-OS and 111-OS). In order to remove any final traces of dust and bubbles, it was found to be very important to centrifuge the cells. Special elastic adapters were made from Dow Corning 3120 RTV Encapsulant by pouring the encapsulant into a cellulose nitrate ultracentrifuge tube into which had been inserted an aluminum mold of exactly the same shape and only slightly greater dimensions (by .2mm) than the cell. This mold was pre-treated with Dow Corning 7 compound, a silicone releasing agent, so that it would easily slip out of place after the encapsulant had hardened. Using the holders made in this way, we were able to centrifuge the cells at centrifugal fields of up to 15,000 g without damage. Samples which were filtered and then centrifuged in this way showed no evidence of particulate scattering.

The light scattering apparatus employed is a conventional light beating spectrometer (1,2). The laser used was a Spectra Physics Model 125A He-Ne laser. The spectrum of the photocurrent was measured with a SAICOR Model SAI-51B time compression spectrum analyzer. The baseline flatness deviation in this instrument (10%) was calibrated and divided out of every measured spectrum before analysis of the data. Both homodyne (self-beat) and heterodyne (in which the scattered light is mixed with an unbroadened component of the incident laser light) techniques were employed. Data were computer analyzed by a conventional least-squares fit to a single Lorentzian curve.

RESULTS AND DISCUSSION

Figure 1 shows a typical light scattering spectrum obtained from a solution of HbCO which was 1.2 mM in heme (solvent: .1M NaCl, .01M BisTris, .003 HCl, pH 7.0). The points are the calibrated output of the spectrum analyzer and the line is the best single Lorentzian curve selected by least-squares analysis to fit the data. The fit is satisfactory, as it should be for hemoglobin, which under these conditions exists almost solely in the tetrameric form. Spectra such as this can be obtained in just a few minutes, so that a complete angular dependence study can be done on a single sample. A typical plot of the variation of the linewidth with $\sin^2(\theta/2)$, the angular factor in K^2, is presented in Figure 2. The points in this

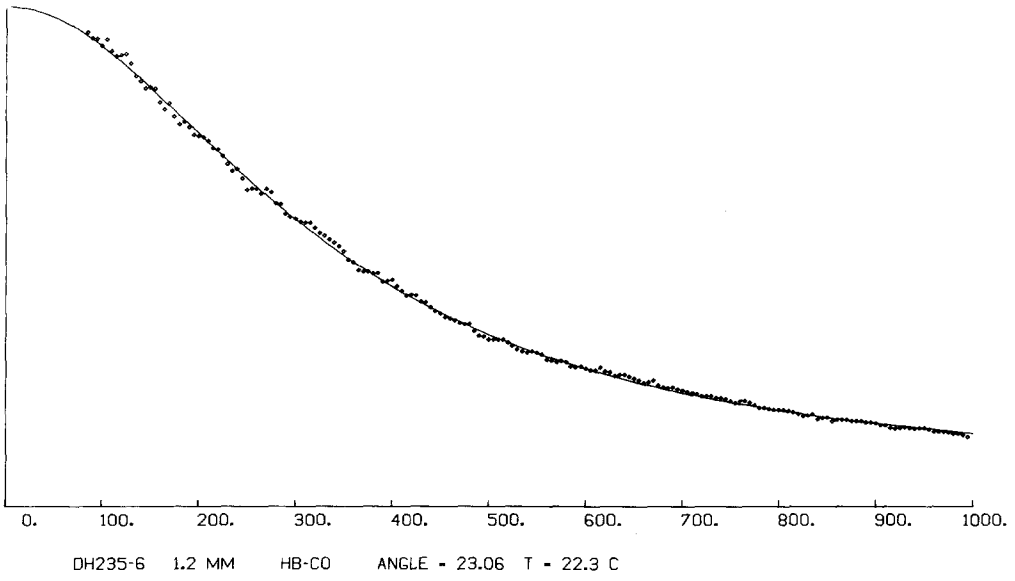

Figure 1. Spectrum of the light scattered from a solution of HbCO (1.2 mM heme, .1 M NaCl, pH 7.0). The horizontal axis is frequency in Hz, and the vertical axis is relative intensity. The points are the data from the spectrum analyzer, and the solid line represents the best Lorentzian fit to the data. The scattering angle was $\theta = 23°$, and the temperature was 22.3°C.

plot are the linewidths obtained at the various angles which were corrected to 20°C and to the viscosity of water. Within experimental error, the data are very well fit by a single line with a zero intercept. The actual intercept in this plot is -8 Hz ± 16 Hz. Under similar conditions, Uzgiris and Golibersuch (3) reported a positive intercept of 53 ± 2 Hz. These two values are incompatible. The slope of the line can be used to calculate the diffusion coefficient of hemoglobin and the value obtained, when properly corrected, is $D_{20,w} = 6.9 \pm .3 \times 10^{-7}$ cm^2/sec. This is in agreement with the value reported by Uzgiris and Golibersuch (3), which was $6.7 \pm .2 \times 10^{-7}$ cm^2/sec.

We have performed measurements such as these on solutions of HbCO and HbO_2 at a number of pH's and protein concentrations, going down by a factor of ten or more in concentration. Under no conditions have we observed an angular dependence which could be interpreted as having a positive intercept. We have done experiments at high scattering angles in the homodyne mode and similarly have observed

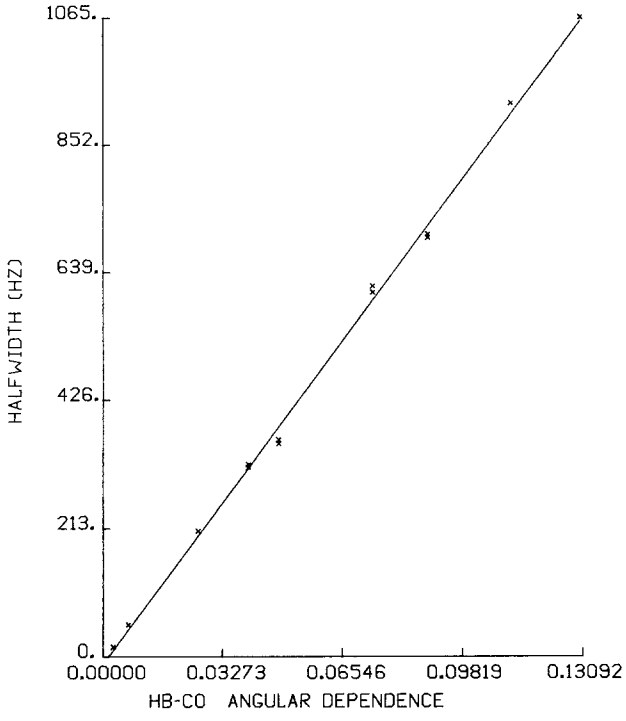

Figure 2. A plot of the light scattering halfwidth in Hz as a function of $\sin^2(\theta/2)$, the angular factor in K^2. The solution is described in the caption to Figure 1. To within experimental error, the data can be represented as a linear function with a zero intercept. The diffusion coefficient obtained from the slope is $D_{20,w} = 6.9 \pm .3 \times 10^{-7}$ cm^2/sec.

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no anomalies in angular dependence. Similar observations in the homodyne mode at high angles have been reported to us by Alpert (7). We have also worked at high pH (up to pH 11) at which the dimer concentration is expected to become higher, and we have not observed angular dependence anomalies.

We have found that our light scattering spectra from hemoglobin solutions can be very anomalous when there is a measurable amount of methemoglobin in solution. We attribute this to the fact that, unlike ferrous hemoglobin, methemoglobin near pH 7 has a strong absorption at 632.8 nm, the wavelength of the He-Ne laser line. Moreover, if a slight amount of particulate matter is added to the solution in these cases, observation of the scattering region through a telescope shows substantial convection caused by the local heating of the solution by

absorption of the laser light. We have therefore adopted the convention that, if a sample has any 630 nm peak in its optical absorption spectrum, we discard it, and if any sample has this absorption after a light scattering experiment is completed, the data are suspect.

One of the confirmations of the kinetics interpretations of Uzgiris and Golibersuch (3) is the fact that the positive intercepts observed are linear in magnitude with concentration. We have observed this same concentration dependence for the heating and convection effects caused by the presence of methemoglobin. Moreover, the kinetics interpretation of the positive intercepts can be reconciled with theory only if the polarizability of the dimer is assumed to be greater than that of the tetramer by a factor of 1.4 to 1.7 (3), whereas it would normally be expected to be half the tetramer polarizability. The high dimer polarizability was inferred from the change observed in refractive index and extinction coefficient as solution conditions were changed to increase the concentration of the dimer, a previously unreported phenomenon. We believe this much more likely to occur if these solution changes enhance the formation of methemoglobin which changes the optical extinction coefficient, and hence the index of refraction at 632.8 nm.

Mansouri and Winterhalter (7) have reported the kinetics of autoxidation of HbO_2 to methemoglobin at 37°C. We have repeated these measurements at room temperature and have found that at 23°C, there is about a 5% conversion of HbO_2 to methemoglobin in 24 hours at pH 7. This 5% level is the maximum tolerable level, particularly at high hemoglobin concentrations, and we have established the practice of discarding all HbO_2 samples within 12 hours of oxygenation. We have also observed the kinetics of autoxidation as a function of incident laser intensity to determine whether the laser is contributing to the oxidation process. It appears that the only contribution of the laser is to raise the temperature slightly, and thus only slightly raise the rate of autoxidation.

In conclusion, we are unable to reproduce the positive intercepts in the plot of linewidth vs. K^2 reported by Uzgiris and Golibersuch. It is, of course, possible

that our samples are contaminated with something which follows normal K^2 dependence, and that the previously reported effect is real. We believe, however, that a much more likely explanation for these positive intercepts is the absorption of the light by methemoglobin and the resulting convection. Workers in this field are cautioned to examine this point carefully when using a He-Ne laser to perform experiments on hemoglobin, particularly HbO_2.

ACKNOWLEDGEMENTS: This work was supported by grants from the National Science Foundation (GP-28495X1) and the American Chemical Society Petroleum Research Fund (2645-G2). DDH is an NIH predoctoral trainee.

References

1. Dubin, S.B., Lunacek, J.H., and Benedek, G.B. (1967) Proc. Nat. Acad. Sci. U.S.A. 57, 1164-1171.

2. Ford, N.C. (1972) Chem. Scripta 2, 193-206.

3. Uzgiris, E.E. and Golibersuch, D.C. (1974) Phys. Rev. Lett. 32, 37-40.

4. Bloomfield, V.A. and Benbasat, J.A. (1971) Macromolecules 4, 609-613, and references therein.

5. Drabkin, D.L. (1946) J. Biol. Chem. 164, 703-723.

6. Benesch, R., Benesch, R.E., and Yu, C.I. (1968) Proc. Nat. Acad. Sci. U.S.A. 59, 526-532.

7. Alpert, S., private communication to be published.

8. Mansouri, A. and Winterhalter, K.H. (1973) Biochem. 12, 4946-4949.