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Hemoglobin Function in the Water–Ethylene Glycol Cosolvent System: Linkage between Oxygen Binding and Hydration†

Robert N. Haire and Bo E. Hedlund*

ABSTRACT: The effects of ethylene glycol (EG) on the oxygen binding properties of human hemoglobin are described in this report. Under the conditions used, the hemoglobin molecule remains in the intact tetrameric form in up to 70% (w/w) EG, corresponding to a mole fraction of EG of 0.4. Interaction between the cosolvent and the hemoglobin is quite weak. Only at high concentrations of EG are the effects on the oxygen binding curve detectable. In the range of mole fraction of EG up to 0.2, oxygen affinity is decreased. In the range of mole fraction of EG between 0.2 and 0.4 (corresponding to molar concentrations of 8–12 M EG), hemoglobin oxygen affinity increases, eventually becoming higher than the value obtained in the absence of EG. Experiments were carried out in the presence of 0.013, 0.10, and 1.0 M NaCl to evaluate the linkage between EG and chloride as allosteric effectors and the possible general effect of ionic strength on oxygen binding properties of hemoglobin in the presence of cosolvent. The effects of EG on hemoglobin ligation are discussed in terms of a model in which EG interacts with hemoglobin in a weak allosteric fashion at the lower concentration range (less than mole fraction of 0.2) while at the higher range (mole fraction of 0.2–0.4) perturbations of protein hydration lead to stabilization of the high-affinity form of hemoglobin.

Hemoglobin is the main protein in the mammalian red cell, present in a concentration of about 30% by weight. The cellular concentration of potassium and chloride ions is maintained within certain levels, and metabolic pathways produce adenosine triphosphate and 2,3-diphosphoglycerate, both of which have well-defined roles in the physiology of the red cell. Thus, 2,3-diphosphoglycerate is an allosteric effector by virtue of its differential interaction with the two conformational forms of the hemoglobin molecule. Such allosteric effects, including the effect of small anions and protons, have received considerable attention both experimentally and theoretically. The theory of linked thermodynamic functions as developed by Wyman (1964, 1965) represents a framework within which allosteric effects can be modeled and described. Structural information about the hemoglobin molecule, including the conformational extremes and the interactions between the protein and its effectors, has been refined to high resolution by means of X-ray crystallography (Fermi, 1975; Baldwin, 1980; Arnone, 1972). Studies of molecular dynamics of proteins in general and heme proteins in particular, using both low-temperature ligation measurements (Austin et al., 1975; Alberding et al., 1978), hydrogen exchange (Englander & Mauel, 1972; Hedlund et al., 1978), and energy minimization calculations (Gelin & Karplus, 1979), have provided another dimension of insight into protein function.

Despite extensive accumulation of experimental data and theory the role of the solvent as a functional component remains poorly understood. From structural, energetic, and dynamic points of view, descriptions of interactions between the protein and surrounding solvent molecules are still primarily qualitative. This is not to say that the general area of protein "hydration" and/or "solvation" has been ignored. Rather, it is the functional aspects of protein–solvent interactions that remain essentially uncharacterized. Thus, in the case of hemoglobin, one may ask if it is possible to define the role protein–water interactions play in the transfer of free energy between the binding sites of allosteric effectors and

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oxygen binding sites at the heme groups. In a discussion of the role of water molecules, identified by X-ray crystallographic measurements to be located between groups in the hemoglobin molecule, Perutz (1977) suggests that these may act as a means to lengthen the distance of hydrogen bonds. In the hemoglobin tetramer, 90 molecules of water seem to be sufficiently stationary to allow observations by X-ray crystallography. This accounts for about 10% of classical hydrodynamic water content (0.3 g of H_2O/g of protein) (Kuntz & Kauzmann, 1974). This distinction between tightly bound (observable by time-average electron density) and loosely bound water is likely to represent extremes of a more or less continuous distribution of average occupancy of a given site. Furthermore, it should be noted in this context that protein crystals often are obtained from highly concentrated solutions of salts. In these solutions the water activity and therefore the interaction between protein and water are likely to be perturbed.

Although the equilibrium between hemoglobin and its solvent environment is likely to be of functional relevance, the effect of changes in water activity on hemoglobin oxygen binding is normally obscured by allosteric effects of ionic species in solution. Hemoglobin functions quite well in the absence of salt, exhibiting a high oxygen affinity and a slightly decreased cooperativity compared to the value obtained in the presence of salt (Haire & Hedlund, 1977). In moderate ionic strength (0.1 M) hemoglobin is balanced at the midpoint of the chloride allosteric effect. At molar concentration of salt the position of the binding curve is shifted further toward low affinity, but the shape of the curve remains essentially invariant. Functional consequences of changes in water activity in these solutions have been masked by the specific and powerful allosteric chloride effect. A potentially more useful approach toward probing energetic aspects of the interaction between hemoglobin and its aqueous environment is to partially replace water with a nonaqueous solvent in which the protein remains functional. We have measured thermodynamic aspects of oxygen binding to hemoglobin in the water–ethylene glycol (EG)[1] solvent system up to 70% EG by weight, which corresponds to a mole fraction of EG of 0.4 and a molar concentration of 12.2 M. At high concentrations of EG, we have altered the concentration and activity of water to a considerable extent without greatly affecting the functional integrity of the hemoglobin molecule.

This report is intended to test the hypothesis that individual water molecules play an integral part in the function of proteins and that functional parameters can be influenced by change in bulk water activity. Hemoglobin, being a protein that is well characterized from a thermodynamic point of view, is well suited for such a study since its interactions with a number of solutes are well delineated and a theoretical treatment of water activity can be incorporated into established linkage schemes.

Materials and Methods

Human hemoglobin, prepared as previously described (Barksdale et al., 1975), was used without removing minor components. Ethylene glycol (Baker Analyzed Reagent) was used without further purification. Mixtures of solvent, buffer, and hemoglobin were prepared volumetrically. Oxygen binding curves were obtained on a point-by-point basis by using the thin-layer membrane, gas dilution system developed by Dolman & Gill (1978). All measurements were carried out in the presence of 0.05 M Bis-Tris.[1] In all experiments, the hemoglobin concentration was held constant at 1.0 mM tetramer. Three concentrations of chloride were used: 0.013, 0.10, and 1.0 M. The lowest represents the amount of HCl needed to neutralize the buffer. All experiments were carried out at pH 7.0 measured at the temperature of the experiment. According to Sage & Singer (1962), the glass electrode functions adequately for pH measurements in the presence of EG. In the few cases where the final pH was slightly higher (<0.06 pH unit), the resulting P_{50} was adjusted by using the normal Bohr coefficient. Data were collected at 5, 15, and 25 °C. At the highest temperature, and at high concentrations of EG, there was evidence of some hemichrome formation which could be minimized by carrying out the experiment as rapidly as possible.

Oxygen solubility was determined by the couloximetric method (Haire et al., 1977). Changes in oxygen solubility as a function of EG were related to oxygen solubility in pure water. Thermodynamic parameters for oxygen solubility were based on these measurements and the very precise oxygen solubility measurements in water reported by Benson and collaborators (1979). Results obtained were in excellent agreement with solubility measurements of argon in the same cosolvent system (Ben-Naim, 1968).

Results and Discussion

Properties of the H_2O–EG Cosolvent System. Water and ethylene glycol differ in terms of certain properties but are quite similar in others. Although density and viscosity are higher for pure EG than for pure water, dielectric constant and solubility of small gaseous compounds are approximately the same. Although recent studies by Beece et al. (1980) suggest that solvent viscosity is an important, possibly dominant variable, in the rate of reaction between heme proteins and ligands, we assume here that the relatively modest change in viscosity (the relative viscosity $\eta/\eta_0 \approx 7$ at a mole fraction of EG of 0.4) will have a minimal effect on equilibrium constants. Thus, the influence of viscosity on the ratio of "on" and "off" constants is probably less than the effect of either measured independently. Furthermore, Bernard et al. (1975) report that the rate constant of CO binding is unaffected by the presence of 50% EG at 0 °C. The interaction between water and ethylene glycol results in heats of dilution which are large and endothermic. The excess heat of dilution is considerable (85–90 kcal/mol) (Savage & Wood, 1976). Water, therefore, interacts very strongly with dissolved ethylene glycol. The solubility of gases referred to above represents another approach in comparing thermodynamic properties of pure water, pure EG, and mixtures of the two solvents. The studies of Ben-Naim and collaborators (Ben-Naim, 1968, 1980; Yaacobi & Ben-Naim, 1973) on the solubility of argon in a number of solvent systems, including the H_2O–EG system, provide a thermodynamic framework for the evaluation of solvent properties. Although the free energy of argon and oxygen solubility at 25 °C are nearly identical in water and EG, the enthalpy, entropy, and heat capacity differ greatly. Within the context of this report, it suffices to summarize gas solubility in the H_2O–EG system as follows:

(I) The solubility of argon and oxygen in pure water at 25 °C is characterized by a relatively large enthalpy (about 3 kcal/mol), an entropy of 15–20 eu, and a heat capacity (ΔC_p) of 40–50 cal/(mol K).

(II) Gas solubility in other polar solvents (alcohols, polyols, dioxane, etc.) is characterized by lower enthalpy, lower entropy, and a small heat capacity.

[1] Abbreviations: EG, ethylene glycol; Bis-Tris, [bis(2-hydroxyethyl)amino]tris(hydroxymethyl)methane.

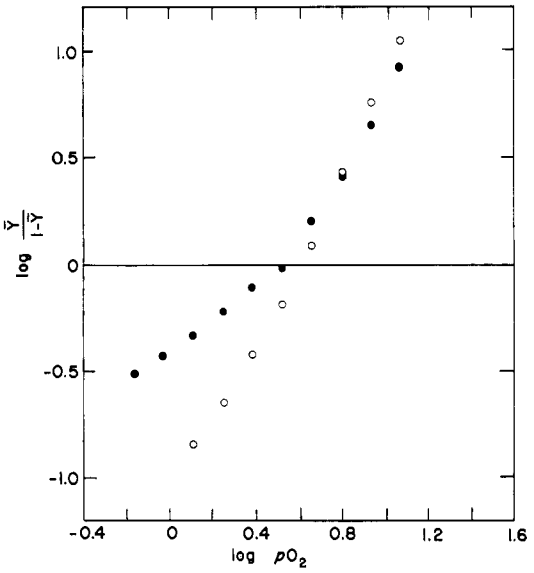

FIGURE 1: Time-dependent changes in hemoglobin oxygen binding curves obtained in the presence of high concentration of EG (mole fraction = 0.48; 76% w/w). Both were carried out under identical conditions (see legend of Figure 2) with the exception of the time allowed for equilibration at each point. The points illustrated by the open symbols represent equilibration for about 3 min in the gas mixing cell, while the closed symbols represent 10-min equilibration at each point. Ten to twelve gas dilution steps are carried out in each instance. The symbol $\bar{Y}$ refers to average degree of ligand saturation of the four heme groups.

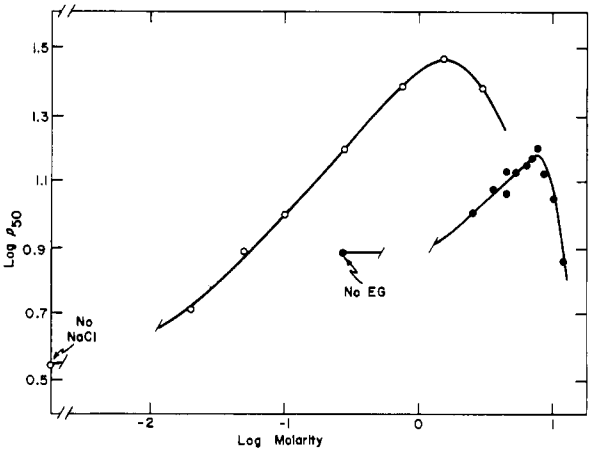

FIGURE 2: Changes in oxygen affinity of human hemoglobin as a function of concentration of NaCl (open symbols) and ethylene glycol (filled symbols). Chloride data, previously published (Haire & Hedlund, 1977), were obtained at pH 7.4 at 37 °C in the absence of buffering ions. Data collected in the presence of EG were obtained at 25 °C in the presence of 0.05 M Bis-Tris, pH 7.0, and 0.1 M chloride. Hemoglobin concentration was 1.0 mM tetramer in both sets of experiments.

(III) In the H_2O–EG mixture, the thermodynamics of gas solubility changes from water characteristics to EG characteristics between mole fractions of EG of 0 and 0.3. Therefore, at higher mole fraction of EG, the solvent mixture resembles a "conventional" solvent.

As stated, studies carried out in this laboratory on the solubility of oxygen in the H_2O–EG mixture agree well with Ben-Naim's results with argon. In general, the solubility parameters of oxygen closely mimic those of argon (Vilhelm et al., 1977).

Structural Integrity of Hemoglobin in the Presence of EG. Human hemoglobin was stable for hours at room temperature in the presence of up to 60% EG. Above this concentration time-dependent changes in the distribution of hemoglobin species were observed, indicative of formation of methemoglobin followed by irreversible denaturation. As an example of such changes, Figure 1 illustrates the oxygen binding of hemoglobin in the presence of 76% (w/w) EG. One curve was obtained over a 2-h period and the other as rapidly as possible (within 20–30 min after mixing the solutions).

The thermal stability of proteins is not greatly affected by relatively high concentrations of EG. Back and collaborators (1979) present data showing that the T_m (the temperature of the maximum rate of denaturation) of ovalbumin is decreased somewhat (i.e., the protein is destabilized) by 28% EG, while triols and larger polyols tend to slightly stabilize the protein. A similar study by Gerlsma & Stuur (1972) indicates that the effect of EG on T_m is very small for both lysozyme and ribonuclease, amounting to a few degrees even at 5 M EG. Furthermore, the effect is not strongly dependent on the pH of the experiment. These experiments suggest that EG does not interact in a preferential fashion with either the native or the denatured form of the protein. From a thermodynamic point of view the protein behaves nearly the same in a 5 M solution of EG as in pure water. However, it should be kept in mind that even the rather concentrated solutions used in the two studies referred to above (i.e., 28% and 5 M, respectively) correspond to a mole fraction of approximately 0.1, a concentration of solute which is sufficiently low not to greatly alter the water concentration (15% or less). In this study of hemoglobin *function* (as opposed to structure), we go well beyond this change in water concentration in that molar concentrations of water as low as 20 M are obtained, corresponding to a decrease in water concentration of 60%. Even so, the changes in functional properties are relatively subtle which in turn suggests that the effect of more dilute solutions of EG on the *structural* integrity of proteins in general and hemoglobin in particular should be modest indeed. However, it appears that in extreme conditions, i.e., in the presence of EG at a mole fraction of 0.5 or higher, the forces responsible for retaining three-dimensional structure of proteins begin to become sufficiently perturbed to lead to partial denaturation of proteins in solution (Sage & Singer, 1962; Tanford, 1968, 1969). Studies of the structure of selected proteins crystallized at low temperature in the presence of high concentration of EG may provide clues toward elucidating which specific solvent–protein (water–protein) interactions are critical in terms of native structure of the protein.

Changes in Oxygen Affinity of Hemoglobin in the Presence of EG. The normal way to illustrate the effect of an allosteric ligand on the oxygen affinity of hemoglobin is to plot log P_{50} or log P_m (expressed in mmHg) against the concentration of the allosteric ligand, expressed in a linear or logarithmic fashion. The relative effects of EG and chloride ion on the oxygen binding curve of hemoglobin have been illustrated in Figure 2. Change in oxygen affinity has been plotted against molar concentration of the solute with both variables being expressed in logarithmic scale. The total effect exerted by EG on the oxygen binding properties of hemoglobin is relatively small in comparison with the chloride effect. Second, the EG effect occurs at very high molar concentrations of solute, concentrations sufficiently high to significantly alter the concentration of water. Oxygen binding curves measured at 25 °C in varying concentrations of EG are illustrated in Figure 3. These measurements were carried out in the presence of 0.1 M chloride. The concentration of EG is here expressed in mole fraction to better indicate change in concentration of both solvent components. The general pattern observed at all temperatures is first a decreased affinity followed by increased

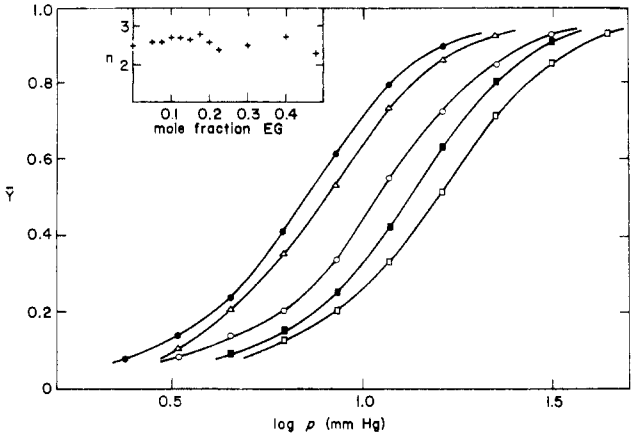

FIGURE 3: Representative oxygen binding curves obtained at 25 °C for various concentrations of EG. The following mole fractions are illustrated: 0 (△); 0.125 (■); 0.20 (□); 0.3 (○); 0.4 (●). The inset illustrates the variation in Hill's coefficient n for the oxygen binding curves obtained at 25 °C.

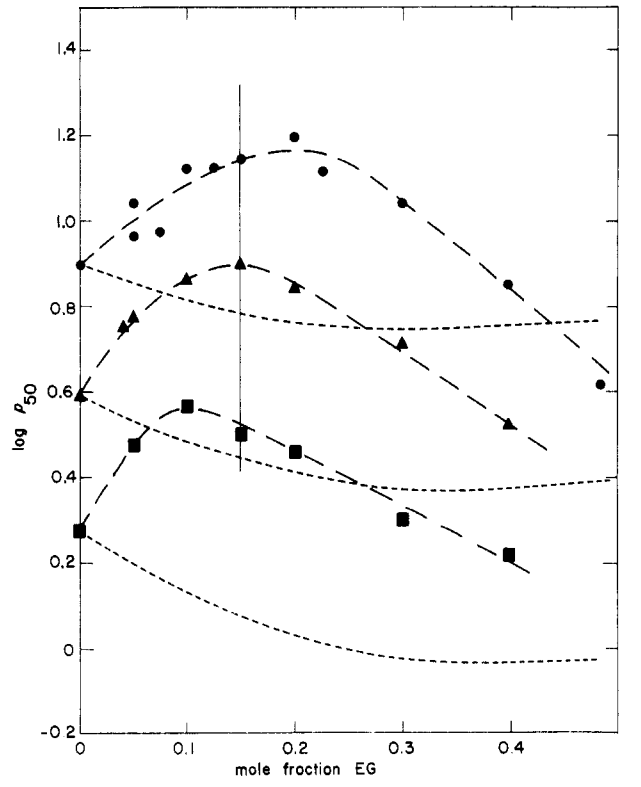

FIGURE 4: Broken lines connecting the experimental points illustrate the dependence of oxygen affinity upon mole fraction of EG at 25 (●), 15 (▲), and 5 °C (■) expressed in mmHg. All experiments were carried out in 0.1 M Cl^-. The vertical line at a mole fraction of EG of 0.15 illustrates the change of position of the point of maximal decrease in oxygen affinity. The dotted line originating from the ordinate shows the relative change in oxygen solubility due to the presence of EG by using EG-free buffer as the base line. The change in solubility is expressed as the change in molar concentration of oxygen on a logarithmic scale. Thus, so that oxygen binding to hemoglobin can be expressed in terms of $[O_2]_{50}$, i.e., the molar oxygen concentration at half-saturation, the dotted lines represent the magnitude of correction that has to be applied to binding data expressed in terms of partial pressure of oxygen. The maximum correction amounts to 0.30 log unit at 5 °C, mole fraction of EG = 0.4. Therefore, at 5 °C the oxygen solubility in this EG–H_2O mixture is 50% of that of water. The experimental points illustrated have not been corrected for change in oxygen solubility.

oxygen affinity at higher concentrations of EG. Although there is a slight decrease in cooperativity at high EG concentration (see inset, Figure 3), the magnitude of this effect

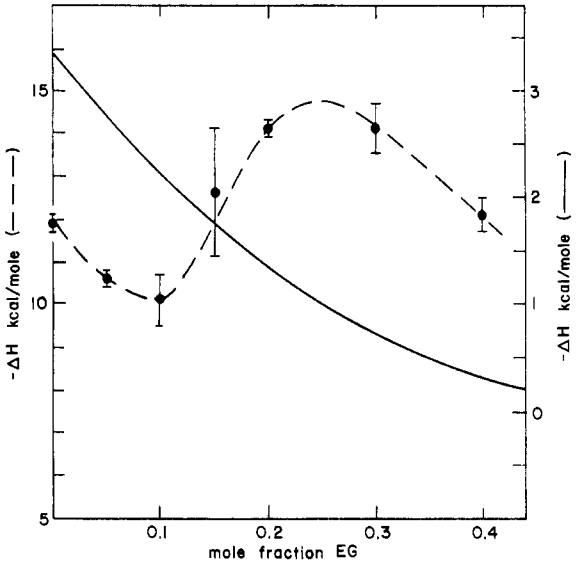

FIGURE 5: Enthalpy of oxygen binding as a function of EG concentration. The results are calculated based on the P_{50} measurements illustrated in Figure 4. The large error bar obtained at a mole fraction of 0.15 is due to the asymmetry of the three curves in Figure 4. The solid line represents the change in enthalpy of oxygen solubility in EG–H_2O mixtures at 15 °C.

suggests that it is less due to the formation of dimers than to changes in the subunit interface presumably due to relatively subtle alterations in critical electrostatic interactions responsible for maximal cooperativity. A similar magnitude of decrease in cooperativity is observed in the total absence of ions, i.e., when oxygen binding curves are measured in solutions of deionized hemoglobin (Haire & Hedlund, 1977).

A more complete description of the effect of EG on the oxygen affinity of human hemoglobin is given in Figure 4. The effect observed in the presence of EG is qualitatively the same at the three temperatures employed. The change from decreasing to increasing oxygen affinity occurs at lower mole fraction with lower temperature. The broken lines in Figure 4 illustrate the relative change in oxygen solubility over the range of EG concentrations used. Since only three temperatures were employed, the enthalpies obtained by van't Hoff analysis are approximate. However, since this type of analysis has been utilized by Anusiem & Oshodi (1978) in a related study (further discussed below), a similar analysis of the results presented in this paper does provide a basis for comparison. The van't Hoff data based on the data presented in Figure 4 have been illustrated in Figure 5. Reasonable least-squares fits are obtained, with the exception of the enthalpy obtained at a mole fraction of EG of 0.15. The solid line at a mole fraction of 0.15 (in Figure 4) illustrates the reason for the large error in enthalpy obtained at this particular point.

The experiments illustrated in Figures 3 and 4 were carried out in the presence of 0.10 M chloride ion which is near the midpoint of the chloride allosteric effect. For evaluation of the possible linkage between EG and chloride, a number of experiments were also carried out in very low (0.013 M) and fairly high (1.0 M) chloride. These experiments were carried out at 25 °C. The results are illustrated in Figure 6. The relative effect exerted by EG on hemoglobin oxygenation is not markedly changed when chloride concentration is altered from 0.013 to 0.10 M. However, a further increase to 1.0 M changes the EG effect significantly. The "low-affinity" part of the effect becomes less notable, and the "high-affinity" tendency at high concentration of EG becomes more pronounced. As a consequence, at a mole fraction of EG of 0.3

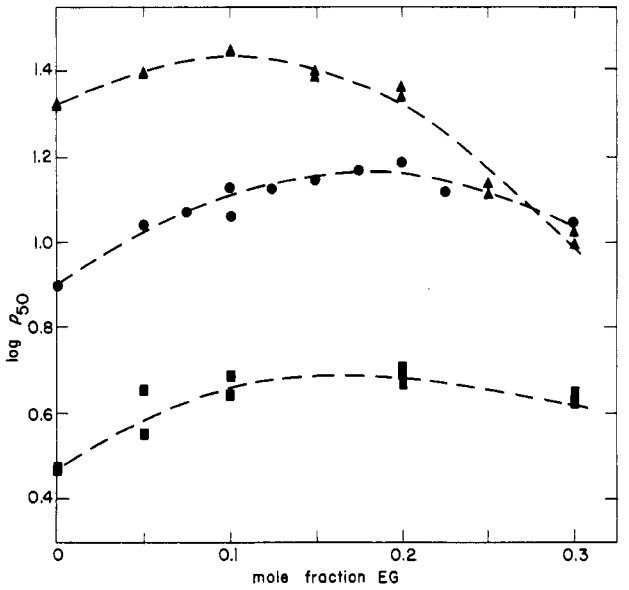

FIGURE 6: Combined effect of EG and chloride ion on the oxygen affinity of hemoglobin is illustrated. Oxygen binding curves were obtained in 1.0 (▲), 0.10 (●), and 0.013 M (■) chloride ion. The temperature was 25 °C and the pH was 7.0. Buffer and protein concentration was the same as in other experiments.

there is no additional change in oxygen affinity between 0.10 and 1.0 M chloride. Thermodynamic aspects of linkage between EG and chloride ion are further discussed below.

A number of studies, carried out in other laboratories, pertain to the results presented here. Among these are the extensive studies of hemoglobin oxygen binding in the presence of alcohols as reported by Cordone and co-workers (1979, 1981). The effect of methanol, ethanol, and propanol on the oxygen binding of hemoglobin is similar to the first region of the effect of EG as reported here. Thus, *n*-propanol at a concentration of 2 M decreases the oxygen affinity by about 0.3 log unit. A similar magnitude of change is observed for methanol at about 5 M, while the effect of ethanol occurs at an intermediate concentration. Since further increase in alcohol concentration causes protein denaturation (Herskovitz et al., 1970), it is uncertain whether the influence on hemoglobin oxygen binding exerted by the small alcohols will mimic the effect produced by EG. Another complication in comparing the effect of alcohols with the results obtained with EG is the possibility that the relatively compact alcohol can interact directly with the heme and thus at high concentration act as competitive ligands. In their studies of the interaction between alcohols and ferric heme proteins, Muhoberac & Brill (1980) conclude that both methanol and ethanol bind as weak ligands to the heme group. However, it is likely that the EG molecule is bulky enough to exclude the possibility of direct heme binding.

Another study of particular relevance to this report is the investigation carried out by Anusiem & Oshodi (1978). They measured the effect of EG on the association between azide and human methemoglobin. In the range of EG mole fraction between 0 and 0.4, they observe a modest decrease of the association constant for azide amounting to approximately a factor of 2. The enthalpy profile indicates a maximum at a mole fraction of EG near 0.2. The total change in enthalpy is approximately 3 kcal over this range. At higher concentrations of EG (>0.4) more dramatic effects are observed, which may be due to the formation of hemichrome-type compounds. Although similarities between the behavior of azide–ferrihemoglobin and the behavior of oxygen–ferrohemoglobin are not necessarily expected, certain features of the former study do support the dual effect of EG. Of particular importance is the observed enthalpy profile, with a maximum at a mole fraction of EG of 0.18. Detailed comparison is complicated by two factors influencing the binding of azide to methemoglobin. One of these is the effect of electrostatic factors on the binding of an anion to ferrihemoglobin, and the other is the equilibrium between two different spin forms of methemoglobin, the equilibrium being dependent on the EG concentration. Thus, quantitative comparison is probably not valid.

An Approach toward the Analysis of the Linkage between Oxygen and Chloride Binding and the Combined Effect of Two Solvent Components. The effect of EG on hemoglobin oxygen affinity which occurs at lower concentration (<8 M) can in principle be treated in allosteric terms. The effect, as illustrated on a molar scale of ligand concentration in Figure 2, is a rather weak one, since it occurs at very high "ligand" concentration and the total change in Gibbs free energy is much smaller than that observed with ionic effectors. For analysis of both the allosteric effect and the solvent perturbation effect occurring at higher EG concentration, the molar concentration scale employed in Figure 2 is not an ideal choice. Instead, mole fraction is used as a measure of relative activity of the two solvent species, since vapor pressure measurements indicate the H_2O–EG cosolvent system closely follows Raoult's law (Trimble & Potts, 1935). Furthermore, from a practical standpoint, the range of mole fraction of EG between 0.1 and 0.4 becomes very steep in a molar or log molar scale, while in a mole fraction or log mole fraction, the experimental results become easier to illustrate (compare Figure 2 and Figure 4).

In general, the effect of an allosteric ligand (X) in hemoglobin oxygen affinity can be expressed in the manner

$$\frac{\mathrm{d} \ln K}{\mathrm{d} \ln a_X} = \Delta\nu_X \qquad (1)$$

where the equilibrium constant K (in mmHg) refers to the binding of oxygen to hemoglobin and a_X is the activity of ligand X. The quantity $\Delta\nu_X$ represents the difference in the number of oxygen-linked ligands bound between the deoxygenated and oxygenated forms of the protein. This expression is often substituted by the equation

$$\frac{\Delta \ln K}{\Delta \ln c_X} = \Delta\nu_X \qquad (2)$$

where c_X refers to the concentration of ligand X. In this report we routinely use changes in log P_{50} as estimates of log K. Since there is little change in the shape of the oxygen binding curve under different conditions, changes in log P_{50} are nearly identical with changes in log P_m.

For illustration of the change in oxygen affinity as a function of the logarithm of mole fraction of EG, the results at 5 and 25 °C in the presence of 0.1 M salt illustrated in Figure 4 have been replotted as solid lines in Figure 7. The two sets of data adequately follow the calculated fractional saturation values (for the allosteric effect) of a simple association reaction between a ligand and hemoglobin. At 5 °C the best fit of an EG "binding site" is obtained with half-saturation at a mole fraction of EG of 0.05. At 25 °C the half-saturation is near a mole fraction of 0.1. In carrying out this fitting procedure of the experimental data, a second estimate has to be made. This pertains to the magnitude of the allosteric effect in terms of Gibbs' free energy. This quantity, the maximal possible change in free energy that the allosteric ligand can produce in the limit of high concentration, has been referred to as ΔG_{max} (Haire & Hedlund, 1977) and amounts to 4.9 kcal/mol of

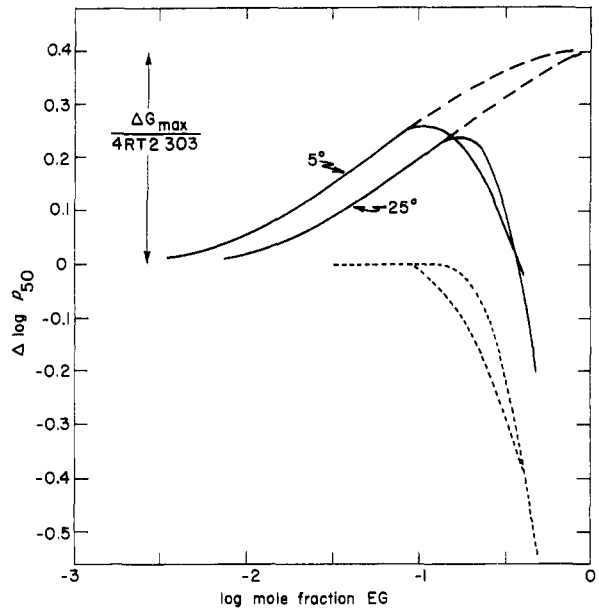

FIGURE 7: Analysis of the linkage between oxygen binding and the dual effects of EG. The solid lines represent actual experimental data (experimental points and scatter are illustrated in Figure 4) on a log mole fraction scale. The broken lines illustrate the theoretical change in oxygen affinity by assuming a simple one-site allosteric equilibrium. Half-saturations of the allosteric effect at a mole fraction of EG of 0.05 and 0.10 at 5 and 25 °C, respectively, and a ΔG_{max} of 2.2 kcal/mol of Hb tetramer yield the best fit to the experimental data. The dotted lines represent the differential hydration effect of EG occurring at high EG concentration at the two temperatures. Summation of the two effects yield the experimental line.

hemoglobin tetramer in the case of chloride. In the case of ethylene glycol as an allosteric effector, this quantity is considerably smaller, estimated to be 2.2 kcal/mol of hemoglobin tetramer under these conditions. This corresponds to a maximal change in oxygen binding affinity of 0.4 log unit in P_{50} (see Figure 7). The actual fitting procedure was carried out by using an iterative graphical procedure in which the two variables, the binding constant defining the half-saturation point and the quantity ΔG_{max}, were varied to yield a best fit through the experimental points.

The midpoint of the allosteric effect and ΔG_{max} are likely to be dependent on experimental conditions, i.e., temperature, pH, and the concentration of ionic effectors. For a more complete description of the interdependence of the Bohr effect, ionic allosteric effects and the weaker allosteric effect produced by ethylene glycol additional linkage relationships must be investigated. Thus, linkage functions such as

$$\left(\frac{\partial \ln K}{\partial \ln a_{H^+}}\right)_{EG,Cl^-} = \Delta\nu_{H^+} \quad (3a)$$

$$\left(\frac{\partial \ln K}{\partial \ln a_{Cl^-}}\right)_{EG,H^+} = \Delta\nu_{Cl^-} \quad (3b)$$

must be studied in order to separate these functions. Equation 3a pertains to the effect of a given concentration of cosolvent on the Bohr effect while eq 3b deals with the effect of chloride, again measured at a given concentration of cosolvent.

The results illustrated in Figure 6 can be utilized to evaluate the effect of EG on the chloride allosteric effect (eq 3b). Table I illustrates how the upper end of the chloride effect (between 0.10 and 1.0 M) is abolished in high concentration of EG, while the low end (0.013–0.10 M) remains largely unaffected.

In summary, the initial effect observed at lower concentrations of EG can be adequately described as a preferential

Table I: Chloride Allosteric Effect as a Function of EG Concentration

mole fraction of EG	Δ log P_{50} at 0.013–0.10 M Cl^-	Δ log P_{50} at 0.10–1.0 M Cl^-
0	0.43	0.42
0.05	0.47	0.36
0.10	0.47	0.46
0.20	0.50	0.17
0.30	0.41	−0.03

association of this compound with the deoxygenated form of hemoglobin. The midpoint of the change in oxygen affinity occurs at a mole fraction of 0.05 and 0.10 at 5 and 25 °C, respectively. The total maximal change in free energy of oxygen affinity can be estimated to 2.2 kcal/mol of hemoglobin tetramer of which approximately 1.5 kcal/mol is observed experimentally. As a comparison, the allosteric effect exerted on the hemoglobin tetramer by a small anion, e.g., chloride, has a midpoint at a mole fraction of 0.002 and alters the oxygen affinity of hemoglobin by nearly 5.0 kcal/mol. In low chloride concentration, the EG effect remains virtually unchanged, while in the presence of 1.0 M salt, the initial part of the EG effect, here treated as a weak allosteric effect, is greatly diminished. The combined effect of high concentrations of both chloride and EG causes the second phase of change in oxygen binding (characterized by increasing affinity) to occur at a lower mole fraction of EG. This is consistent with the hypothesis that this latter effect on hemoglobin oxygen binding is mediated by a change in water activity caused by the presence of both solutes. In analyzing this effect, proposed to be secondary to changes in water activity, and thus involving protein–water interactions, it is necessary to separate this phenomenon from the allosteric effect. Such a separation has been carried out in Figure 7. Although the assumptions discussed above also influence this type of deconvolution, the overall picture will not be strongly dependent on these assumptions. The "differential hydration" effect differs from the "allosteric" effect in two important aspects. First, it is of opposite sign in terms of the observed change in free energy of oxygen binding. Second, the magnitude, as measured by the slope of the dotted lines in Figure 7 illustrating the hydration effect, increases steadily with increasing "ligand activity". This observation is the experimental basis for a model in which the observed increase in oxygen affinity at high concentrations of EG differs from the effect produced by the differential interaction (i.e., "binding") of an allosteric ligand and instead is due to altered hydration. The decreased water activity leads to stabilization of the oxygenated form of hemoglobin, and the degree of stabilization as measured by the maximal *observed* change in oxygen affinity is considerable in terms of free energy (approximately 3 kcal/tetramer). At higher cosolvent concentration, further perturbation of the ferrous hemoglobin structure causes formation of hemichrome followed by denaturation.

Since the steepness of the slope Δ log P_{50}/Δ log a_X is a reasonable measure of change in free energy as a function of change in solvent composition, Tanford's (1969) extension of Wyman's (1965) scheme can be applied to the present case. These treatments incorporate specific stoichiometry for ligands including solvent components. The Wyman–Tanford type analysis of solvent effects does allow comparison with classical-linked functions (protons, chloride, etc.), and although the stoichiometry approach may not be an ideal choice for this system, such an analysis will nevertheless be useful in developing alternative analytical approaches. Thus, by assigning

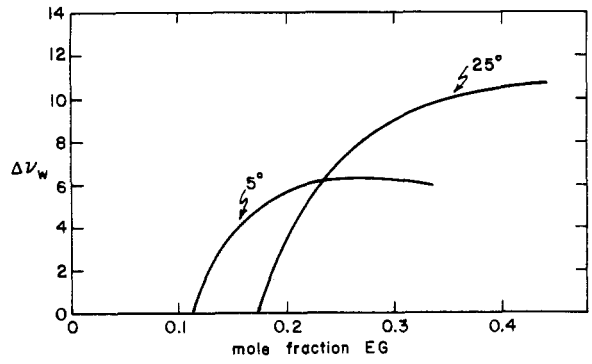

FIGURE 8: Calculated interdependency of $\Delta\nu_w$ and $\Delta\nu_X$ using eq 4 assuming $\Delta\nu_X = 1$. The value of d ln K/d ln a_X was obtained by graphical estimate of the slope of the two dotted lines in Figure 7. Further details are given in the text.

a value to one of the components (in this case $\Delta\nu_X$, the number of linked EG binding sites), we can calculate the resulting change in $\Delta\nu_w$, the number of linked water "sites". If the value of $\Delta\nu_X$ is set to +1, i.e., the value used to analyze the allosteric effect, the resulting values of $\Delta\nu_w$ can be calculated by using the Tanford expression:

$$\frac{\text{d ln } K}{\text{d ln } a_X} = \Delta\nu_X - \frac{n_X}{n_w}\Delta\nu_w \qquad (4)$$

In eq 4, n_X and n_w refer to the mole fraction of solvent component X (here EG) and water, respectively. The dependence of $\Delta\nu_w$ on mole fraction of EG is illustrated in Figure 8. The oxygen linked binding of EG is associated with positive values of $\Delta\nu_w$, amounting to 6 and 10 mol of water/mol of EG at 5 and 25 °C, respectively. This ratio of EG to water is considerably below that present in the cosolvent mixture in the experimental range (mole fraction of EG of 0.3–0.4), suggesting preferential hydration of the deoxygenated form of the protein. Whether this theoretical result can be experimentally tested remains to be seen. However, differential refractometry studies of this system have been initiated in order to further characterize the hydration of hemoglobin in the water–EG cosolvent system (Timasheff & Inoue, 1968). Information from such studies will provide a measure of free energy of "preferential hydration" which will allow analysis of the degree of linkage between this variable and hemoglobin oxygenation. The long-range goal of such studies will be to apply multicomponent theory of differential solvent interaction to functional properties of heme proteins.

A basic question arising from the results discussed in this report pertains to the generality of the effect caused by altering the activity of water surrounding the hemoglobin molecule. Thus, can other solutes (assuming their tendency to denature, oxidize, and dimerize the hemoglobin molecule is not limiting in terms of their ability to compete with the protein for water) produce effects similar to those observed with EG? Relatively few solutes satisfy the criteria outlined, and ethylene glycol may be rather unique in this respect. However, there is some evidence that certain ionic solutions produce a similar effect at sufficiently high concentration. Thus, in the presence of both sodium chloride (Haire & Hedlund, 1977) and phosphate (unpublished observations from this laboratory) an increase in oxygen affinity is observed at high concentration of the ionic solute. Similar results have also been obtained by Amiconi et al. (1981). We have shown that increase in oxygen affinity is not related to dimer formation which occurs in the presence of certain ionic solutes, such as potassium iodide. Instead, it is likely that a phenomenon similar to the effect observed with EG also occurs in the presence of these ionic solutes. Ionic hydration forces lead to the extraction of water from the protein at a much lower solute concentration than in the case of EG. The relative effect of EG vs. ionic solutes on the solubility of an inert compound (the salting-out effect) is a means by which relative changes in water structure due to the introduction of a given solute can be estimated. The Setchenow constant (S) is a measure of the change in solubility of a gas, e.g., oxygen or argon, in a 1.0 M solution of a compound in comparison with the solubility in pure water expressed as the logarithm of the solubility ratio ($S = \log k_0/k$). The ratio of Setchenow constants for EG, NaCl, and sodium phosphate (pH 7.0) is approximately 1:8:16 or in terms of moles of particles 1:4:8. Our studies with chloride and phosphate indicate that hemoglobin oxygen affinity begins to increase at approximately 2 M sodium chloride and 1 M sodium phosphate, while in the case of EG this phenomenon occurs at higher concentration (4–8 M). Thus, sufficient alteration of water activity due to the presence of high concentration of any solute or combination of solutes will lead to changes in a number of specific structural roles that water molecules play in the three-dimensional network of the hemoglobin molecule. Furthermore, we suggest that while a given perturbation may stabilize either of the two conformational extremes of hemoglobin, the sum of these alterations appears to stabilize the oxygenated form of hemoglobin, thus leading to an increased oxygen affinity. On the basis of crystallographic studies on horse methemoglobin (Ladner et al., 1977) and human deoxyhemoglobin (Fermi, 1975), Perutz (1977) has evaluated the role of water molecules as structural entities. Whether a substitution of one or more of specific, interior (high occupancy) water molecules can be traced down as critical in the retention of balance between the two forms of human hemoglobin remains to be seen. A second possibility, in which the role of water is more global, concerns the role of water in the protein hydration shell, a thin layer of water molecules surrounding the protein. In recent years, our knowledge about structural and dynamic aspects of surface "bound" water has increased primarily due to crystallographic studies of relatively small proteins, such as rubredoxin (Watenpaugh et al., 1978) and myoglobin (Phillips, 1980). One may assume that with the continuous development and refinement of X-ray crystallographic techniques it may be possible to assign specific functional roles for water molecules observed in the interior of proteins. Hemoglobin and variants of this protein represent particularly suitable systems for the probing of the role of solvent molecules as structural and functional entities. The crystallographic picture of hemoglobin that has emerged with increasing resolution represents a dehydrated crystal, since crystallization procedures normally involve concentrated solutions of salts or other solutes which greatly affect water activity. A number of functionally important water–protein interactions are, therefore, likely to be affected. A combination of functional studies with difference Fourier analysis as a function of water activity may provide information regarding specific sites of water occupancy where degree of occupancy can be correlated with specific functional parameters. A complete description of such water molecules in terms of structure, energetics, and dynamics represents a highly complex problem in terms of both defining solvent–specific interactions in terms of protein function and the inherent conceptual difficulty in developing possible experimental and theoretical avenues of probing such interactions.

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Registry No. EG, 107-21-1; oxygen, 7782-44-7; chloride, 16887-00-6.

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