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The effect of organic cosolvents on the oxygen affinity of fetal hemoglobin

Relevance of protein-solvent interactions to the functional properties

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We have studied the effects of organic cosolvents (monohydric alcohols and formamide) on the oxygen affinity of human fetal hemoglobin stripped of phosphates and have compared them with the effects of the same cosolvents on the oxygen affinity of human adult hemoglobin under the same experimental conditions. Our results confirm that, in fetal hemoglobin, the T $\rightleftharpoons$ R conformational equilibrium is more displaced toward the T conformation than in the adult form and indicate that increased electrostatic and hydrophobic protein-solvent interactions contribute to this effect. The data reported are discussed in terms of the known amino acid substitutions between the β- and γ-chains and an attempt is made to rationalize the results with a molecular mechanism based on the crystallographic structure of fetal deoxyhemoglobin.

1. Introduction

The oxygen affinity of fetal hemoglobin (HbF) is, in red cells, greater than that of adult hemoglobin (HbA). This effect, which is physiologically relevant, is mainly caused by the fact that HbF, due to the H21(143)His $\beta \rightarrow$ Ser γ substitution, binds organic phosphates less effectively than HbA [1]. However, when isolated and stripped of phosphates, the oxygen affinity of fetal hemoglobin becomes lower than that of the adult form [2,3]. The physiological relevance of this fact is less clear and its physical origin is also not well understood.

Wind et al. [4] suggested that in HbF the deoxy (T) quaternary conformation is markedly stabilized with respect to HbA and that this is the main contributing factor to the reduced oxygen affinity. Two structural explanations have been proposed for the T structure stabilization in HbF: Bonaventura et al. [5] suggested that for stripped HbF the T structure is more stable than for stripped HbA, since the former, in view of the H21(143) His $\beta \rightarrow$ Ser γ substitution, has fewer repelling positive charges in the central cavity; Frier and Perutz [6] pointed out the relevance of the substitutions A4(7)Glu $\beta \rightarrow$ Asp γ and NA3(3) Leu $\beta \rightarrow$ Phe γ that draw the A helix closer to the molecular dyad axis in deoxy-HbF and therefore appear to 'tighten' the T structure. On the other hand, Bianconi et al. [7] studied the local structure of the heme in deoxy-HbF using the technique XANES (X-ray absorption near-edge structure) and attributed the different oxygen affinities of HbA and HbF to subtle differences in the Fe^{2+}-porphyrin stereochemistry between the deoxygenated β- and γ-chains.

In previous studies, however, the interactions of HbF in different quaternary conformations with the solvent and their effect on the conformational and functional equilibria of this protein have not been considered.

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Over the past few years, our group has extensively investigated the role that protein-solvent interactions play in the conformational and functional properties of human adult and bovine hemoglobins [8–19]. This was done mainly by studying the effect of some organic cosolvents (monohydric alcohols and amides) on the oxygen affinity. By analysing the data within the framework of the Monod-Wyman-Changeux (MWC) model [20], it was possible to point out that protein-solvent hydrophobic interactions play a relevant role in determining the $T \rightleftharpoons R$ conformational equilibrium of these hemoglobins.

In order to investigate whether protein-solvent interactions are among the factors that bring about the different oxygen affinity of HbF, we have undertaken this study on the effect of monohydric alcohols and formamide on the oxygen affinity of stripped human fetal hemoglobin. The data indicate that a relevant contribution to the T structure stabilization in HbF arises from increased charge interactions and protein-solvent hydrophobic interactions. An interpretation of this result in terms of the known amino acid differences between the γ- and β-chains is also attempted.

2. Materials and methods

Human fetal hemoglobin was prepared from umbilical cord blood using the following procedure: red cells were washed four or five times with isotonic NaCl and then lysed by adding diethyl ether; the hemolysate was dialyzed overnight against 0.1 M KCl and stripped of organic phosphate by chromatography on Sephadex G-25 fine. HbF was purified by chromatography on CM-cellulose (Pierce) according to the procedure described by Huisman and Jonxis [21]. A linear NaCl gradient in 0.01 M Bis-tris buffer (pH 6.1) was used. No separation between HbF_0 and the minor component HbF_1 was performed. All steps were performed in the cold. After the chromatography, HbF was concentrated to approx. 10% (w/v) and stored at 4° C in the oxy form for no more than 15 days. Methanol, ethanol, *n*-propanol and formamide (Carlo Erba) were used without further purification. Unless otherwise stated, the samples for oxygen equilibrium measurements contained 0.1% (w/v) fetal hemoglobin and 0.05 M Bis-tris buffer (pH 6.9) in H_2O; approx. 15 mM Cl^- was also present. The pH values in the absence of cosolvents were measured with a Corning 240 pH-meter. Oxygen equilibrium measurements were performed by tonometric methods, as already described [8]. The amount of methemoglobin, checked at the end of oxygen equilibrium curves, was found to be less than 5%, even at the highest cosolvent concentrations studied; methemoglobin formation and/or sample denaturation imposed an upper limit to the cosolvent concentrations studied. All measurements were repeated at least twice; to improve the precision of the $\Delta \log P_{50}$ values at low cosolvent concentrations (that are important for the determination of bulk electrostatic contributions, see below), measurements in normal water and at low cosolvent concentration were repeated four or five times. Spectrophotometric measurements were performed with a Varian DMS 90 spectrophotometer interfaced to an Apple Europlus personal computer which performed the acquisition of absorbance values and, on line, the statistical analysis of oxygen equilibrium curves.

The effect of cosolvents on the pH of our buffered solutions has been thoroughly discussed in previous publications from our group [12,16,17]. In particular, it has been shown that alcohols, even at the highest concentrations used, have a negligible effect on pH and that, in contrast, formamide sizeably affects the pH of buffered solutions. The pH dependence of solutions buffered with 0.05 M Bis-tris (pH 6.9 in water) upon formamide concentration has been determined by the method of Douzou [22] and found to conform to the equation:

$$d\,pH/dC_F = -0.24 C_F \qquad (1)$$

where C_F is the concentration of formamide [16]. In order to compare the $\log P_{50}$ values obtained in water or in the presence of alcohols with those obtained in the presence of formamide, these latter have been corrected to compensate for the pH differences. The corrections have been performed by using the data in fig. 1, where $\log P_{50}$ values of

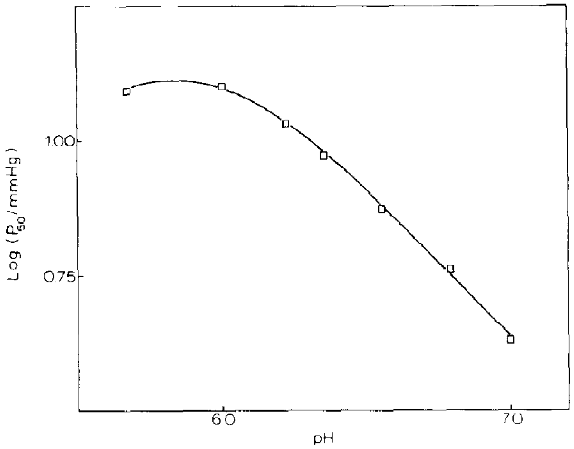

Fig. 1. Dependence of log P_{50} (in the absence of cosolvents) upon pH. $T = 25°$C. All samples contained 0.05 M Bis-tris buffer and 45 mM Cl^-.

HbF (in the absence of cosolvents) are reported as a function of pH. The experimental Δ log P_{50} values measured in the presence of formamide, the pH variations obtained using eq. 1, the Δ log P_{50} corrections due to the Bohr effect (obtained from data in fig. 1) and the corrected Δ log P_{50} values are listed in table 1.

The values of the bulk dielectric constants of water-alcohols and water-formamide mixtures at 25°C were calculated from the data reported by Akerlöf [23] and Rohdewald and Moldner [24], respectively, as already described by Vitrano et al. [16].

Table 1

Δ log P_{50} corrections due to the effect of formamide on pH

[Formamide] (m/l)	Δ log P_{50} [a] (experimental values)	ΔpH [b]	Corrections [c] to Δ log P_{50}	Δ log P_{50} (corrected values)
0.125	−0.01	−0.03	−0.01	−0.02
0.25	−0.03	−0.06	−0.02	−0.05
0.50	−0.05	−0.12	−0.06	−0.11
1.25	−0.15	−0.30	−0.15	−0.30
1.88	−0.24	−0.45	−0.22	−0.46
2.50	−0.32	−0.60	−0.28	−0.60
3.13	−0.41	−0.75	−0.32	−0.73

[a] Experimental conditions as in fig. 2.
[b] Calculated using eq. 1.
[c] Calculated from the ΔpH values (adjacent column) and the data reported in fig. 1.

Optical spectra, in the wavelength range 650–500 nm, were measured with a Cary 118c UV-Vis spectrophotometer interfaced to a PC IBM. Digitized spectra were transferred to an HP1000 computer for off-line analysis. Difference spectra were synthesized by the computer after intensity normalization.

The kinetics of O_2 replacement by CO were followed at $\lambda = 560$ nm with standard stopped-flow techniques. The experiments were performed with the procedure described in ref. 11; the signal from the photometer was sampled and digitized with 12-bit precision at programmable time intervals by a microcomputer-based system that allows acquisition of 255 data points for each run [25]; the experimental data were analyzed in terms of two exponentials of equal amplitude, following Olson et al. [26].

3. Results

Fig. 2 shows Δ log P_{50} values as a function of cosolvent concentration. Here Δ log P_{50} = log P_{50} (C)–log P_{50}(o), where C is the cosolvent concentration expressed in mol/l. As can be seen from fig. 2, alcohols decrease the oxygen affinity (positive Δ log P_{50} values), whilst formamide increases it (negative Δ log P_{50} values). The data in fig. 2 relative to formamide have been corrected for pH effects (see section 2).

Concerning the data in fig. 2, one may wonder whether the effects of cosolvents on the oxygen affinity can be related to those of the same cosolvents on the dimer $\rightleftharpoons$ tetramer equilibrium. To test this point, we have measured the dependence of Δ log P_{50} values upon protein concentration. The data are listed in table 2 and show that, in analogy with previous findings relative to human adult and bovine hemoglobins [16], eventual cosolvent effects on the dimer $\rightleftharpoons$ tetramer equilibrium of HbF do not appreciably affect the experimental results at the hemoglobin concentration used in this paper.

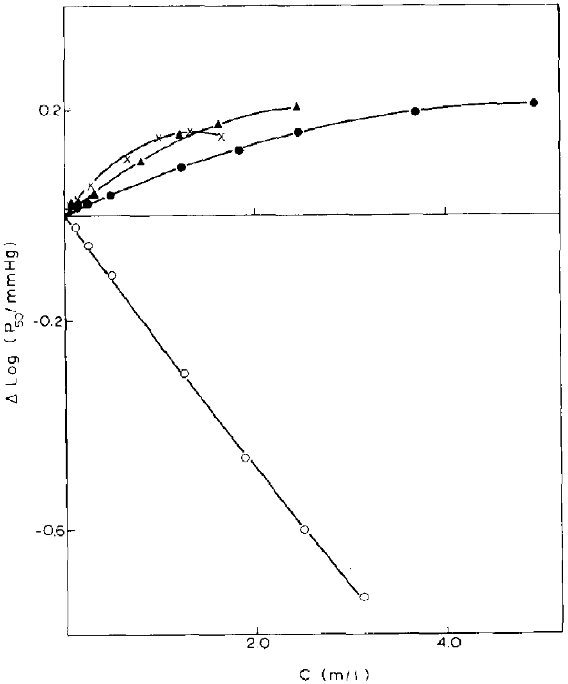

Fig. 2. Δ log P_{50} values as a function of cosolvent concentration. (●) Methanol, (▲) ethanol, (×) n-propanol, (○) formamide. Hemoglobin concentration: 60 μM (in heme). $T = 25\,°C$; 5×10^{-2} M Bis-tris buffer (pH 6.9); 15 mM Cl^-. Data referring to formamide have been corrected for the pH effect, as discussed in the text.

Table 2

Dependence of Δ log P_{50} values upon protein concentration

Solvent	C_{Hb} (M, in heme)	Δ log P_{50}
0.85 M ethanol	6×10^{-6}	0.11
	6×10^{-5}	0.10
	3×10^{-4}	0.10
1.7 M ethanol [a]	6×10^{-5}	0.17
	3×10^{-4}	0.18
1.25 M formamide	6×10^{-6}	−0.16
	6×10^{-5}	−0.15
	3×10^{-4}	−0.16
2.5 M formamide [a]	6×10^{-5}	−0.32
	3×10^{-4}	−0.29

[a] At these high cosolvent concentrations measurements at $C_{Hb} = 6 \times 10^{-6}$ M were not performed since excessive protein denaturation occurred.

According to the MWC model, one has for highly cooperative hemoglobins [27] ($K_R/K_T \ll 1; L \gg 1$):

$$P_{50} = L^{1/4} K_R \tag{2}$$

where L is the usual allosteric parameter and K_R and K_T denote the microscopic oxygen dissociation constants of the R and T state, respectively.

From eq. 2, and considering K_R to be unaffected by the cosolvents used, it follows that:

$$\begin{aligned} 4RT\,2.303\,\Delta \log P_{50} &= 4\,RT \ln P_{50}(C)/P_{50}(o) \\ &= RT \ln L(C)/L(o) \\ &= \Delta G(C) - \Delta G(o) \\ &= \Delta\Delta G(C) \end{aligned} \tag{3}$$

where $\Delta G(C)$ is the standard free energy difference between the R and T states of hemoglobin in the absence of oxygen and in presence of a concentration C of cosolvent.

Two important points need to be considered in more detail. The first concerns the applicability of the MWC model to our system, and the second the assumption that K_R is not affected by the cosolvents used. Experimental evidence bearing on the two above points has been obtained from the functional and spectroscopic properties of HbF.

Fig. 3 shows the dependence of the Hill coefficient (n_H) upon log P_{50}; it is well known [27] that the MWC model predicts the following dependence of n_H upon P_{50}:

$$n_H = 1 + 3\frac{(1 - cP_{50}/K_R)(P_{50}/K_R - 1)}{(1 + cP_{50}/K_R)(P_{50}/K_R + 1)} \tag{4}$$

where $c = K_R/K_T$, as usual. Eq. 4, for constant K_R and K_T, results in a bell-shaped curve when n_H is plotted vs log P_{50}. The continuous line in fig. 3 is the best fit of eq. 4 to the experimental points; as can be seen, the data are fully compatible with the theoretical prediction and the rather good fit suggests that our results can safely be analysed using the MWC model and considering K_R to be not affected by the cosolvents (for further discussion, see the appendix in ref. 16). The MWC parameters obtained from the fitting are reported in table 3; the analogous parameters relative to adult hemoglobin are also reported for

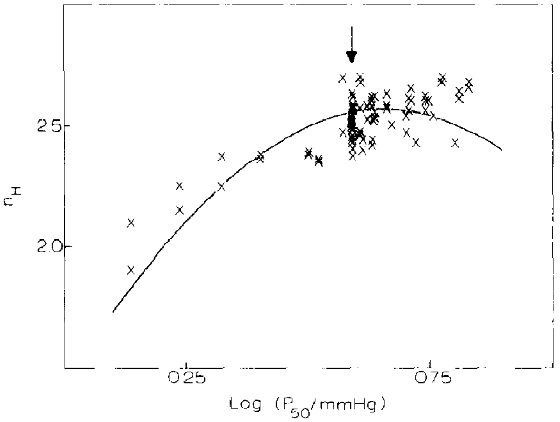

Fig. 3. Hill coefficient (n_H) as a function of log P_{50}. The arrow indicates points obtained in the absence of cosolvent; points relative to measurements in the presence of formamide all fall to the left, those relative to measurements in the presence of alcohols to the right. At each cosolvent concentration oxygen equilibrium curves have been performed at least twice: the n_H values relative to each single measurement have been reported (some of the points are exactly coincident); since the scattering of log P_{50} values is very small, each n_H value has been referred to the average log P_{50} value. The continuous line is the best fit of eq. 4 to the experimental points; the root-mean-square deviation is 0.09 and should be compared with the standard deviation of n_H values (±0.08), estimated from the reproducibility of experimental data in the absence of cosolvents. The log P_{50} values relative to measurements in the presence of formamide are the experimental ones and have not been corrected for the pH effect.

the sake of comparison. It is interesting to note that almost identical values of the parameters K_R and c (and, as a consequence, of the parameter K_T) are found for HbA and HbF; in contrast, the value of the parameter L relative to HbF is almost double that relative to HbA.

Table 3

Monod-Wyman-Changeux parameters for human fetal and human adult hemoglobin

	HbF [a]	HbA [b]
K_T (mmHg)	28.1 ±1	27.8 ±1
K_R (mmHg)	0.73±0.02	0.85±0.02
$c = K_R/K_T$	(2.6 ±0.1)×10^{-2}	(3.0 ±0.1)×10^{-2}
L (in H_2O)	(9 ±0.5)×10^2	(5 ±0.5)×10^2

[a] Experimental conditions: 60 μM hemoglobin (in heme); T = 25°C; 0.05 M Bis-tris buffer (pH 6.9); 15 mM Cl^-.

[b] Experimental conditions: 60 μM hemoglobin (in heme); T = 25°C; 0.05 M Bis-tris buffer (pH 6.9); 15 mM Cl^-; 5 μM catalase. Data taken from table A1 of ref. 16.

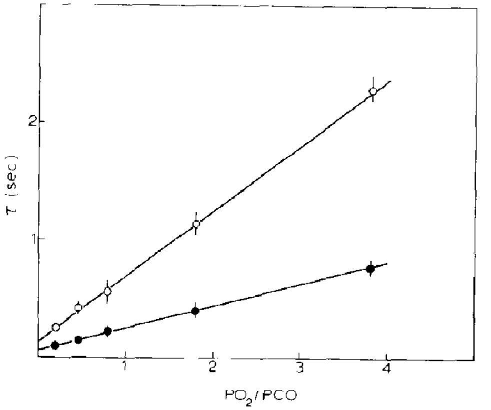

Fig. 4. Time constants of the O_2/CO displacement reaction as a function of the P_{O_2}/P_{CO} ratio. Experimental conditions as in fig. 2. The points represent the average of the experimental values obtained in the absence or presence of cosolvents (1.7 M ethanol or 2.5 M formamide). For each point all experimental values fall within the error bar shown. Open and full symbols refer, respectively, to the slow and fast exponential components of the displacement reaction.

Experimental evidence that the cosolvents used do not affect K_R has also been obtained by studying their effect on the kinetics of O_2 substitution by CO [11]. The results reported in fig. 4 show that, at all the P_{O_2}/P_{CO} ratios investigated, the time constants of the displacement reaction are not affected by cosolvents, thus supporting the assumption that K_R is also unchanged.

Further experimental evidence, although somewhat indirect, for the above assumption can be obtained from visible differential spectroscopy [8,28]. Fig. 5 shows the well-known optical spectrum of oxy-HbF in the wavelength range 650–500 nm; the difference spectra produced by the addition of organic cosolvents are also shown on an expanded scale. It is evident that these difference spectra (except possibly for a small effect produced by high concentrations of n-propanol) are almost vanishing; in contrast, other factors, for example, temperature and inositol hexaphosphate, known to affect sizeably the parameter K_R, do introduce relevant alterations in the visible spectrum of oxyhemoglobin [29,30]. The data in fig. 5 support the assumption that K_R is not affected by

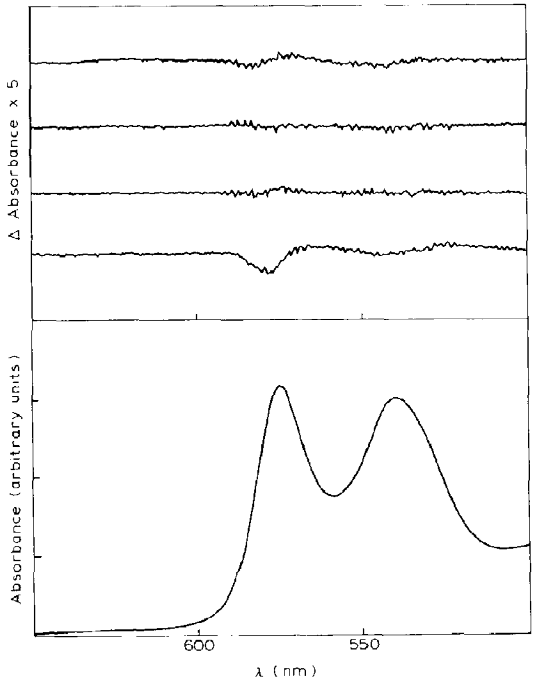

Fig. 5. Difference spectra induced in oxy-HbF by the presence of organic cosolvents. From top to bottom: methanol, 2.5 M; ethanol, 1.7 M; formamide, 2.5 M; *n*-propanol, 1.3 M. The spectrum of oxy-HbF is also shown, in the lower part of the figure, for the sake of comparison.

alcohols and formamide, since they show that these cosolvents do not introduce spectrally detectable distortions at the heme pocket in oxy-HbF.

Using eq. 3 it is possible to relate the observed cosolvent effects on the oxygen affinity ($\Delta \log P_{50}$ values) to their effects on the relative stabilities of the R and T conformations ($\Delta\Delta G$ values). The data in fig. 2 therefore show that alcohols stabilize the T conformation (positive $\Delta \log P_{50}$ and $\Delta\Delta G$ values) whereas formamide stabilizes the R conformation (negative $\Delta \log P_{50}$ and $\Delta\Delta G$ values).

That the cosolvents used do not act as 'classical' allosteric effectors has already been tested in the case of human adult and bovine hemoglobins [8,16] by plotting the quantities $\log[L(C)/L(o) - 1]$ and $\log[L(o)/L(C) - 1]$, for alcohols and formamide respectively, vs $\log(C)$ and by verifying that nonlinear plots are obtained [20]. The same plotting procedure has also been performed in the present case and the results indicate that, indeed, our cosolvents do not behave as allosteric effectors of HbF. Therefore, following the previously used approach [8–19], we interpret the reported results in terms of general solvent effects.

As is well known, the T conformation of hemoglobin is stabilized by electrostatic interactions between charged groups (salt bridges) [31] that become exposed to the solvent following the T → R transition; moreover, it is now well established that the free energy contribution related to this charge displacement is dependent on the value of the bulk dielectric constant of the solvent (16, 17 and 32, and references cited therein). In previous works on the effect of organic cosolvents on the $T \rightleftharpoons R$ equilibrium of hemoglobin, these contributions (bulk electrostatic contributions) have been expressed, in the cosolvent concentration range studied, as:

$$\Delta\Delta G_{\mathrm{bes}}(C) = A\Delta(1/\epsilon) \tag{5}$$

where $\Delta(1/\epsilon) = 1/\epsilon(C) - 1/\epsilon(o)$ and A is a parameter determined by the effective number and the relative positions of surface charges whose interaction free energy is altered following the T → R transition. The value of the parameter A has been found by plotting $\Delta\Delta G$ values vs $\Delta(1/\epsilon)$ and by determining the slope of the straight line common to all the experimental points in the limit $\Delta(1/\epsilon) \to 0$ (see, e.g., fig. 4 of ref. 17). A comment concerning the use of eq. 5 is appropriate. Indeed, the procedure followed to calculate the bulk electrostatic contributions is phenomenological and is not based on any particular theory of electrostatic interactions in protein solutions. It is, however, interesting to note that a linear dependence of the electrostatic free energy upon $1/\epsilon$, for ϵ values ranging between 70 and 90 (i.e., the range of interest in our paper), is obtained from the Tanford-Kirkwood theory [33,34] and from Paul's theory [35], if one uses for the dielectric constant of the protein interior values that are well accepted in the literature (i.e., 2–5).

The plot of $\Delta\Delta G$ vs $\Delta(1/\epsilon)$ for the data relative to HbF is shown in fig. 6. As can be seen, in the limit $\Delta(1/\epsilon) \to 0$ a straight line common to all the experimental points is not well defined. In-

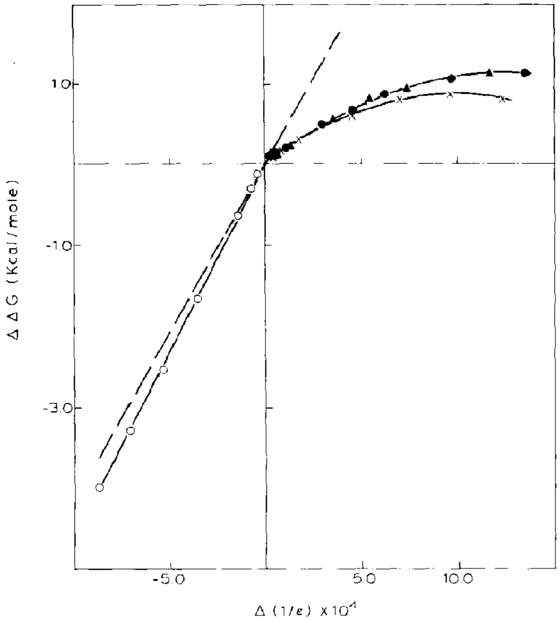

Fig. 6. $\Delta\Delta G$ as a function of the variation of the inverse bulk dielectric constant of the solvent. Symbols and experimental conditions as in fig. 2.

deed, while the data points relative to formamide and to methanol at very low concentrations approach the same linear behavior, in the case of ethanol and n-propanol deviations are observed even at the lowest concentrations examined.

The fact that, contrary to HbA, deviations from the linear behavior are observed even at low concentrations of ethanol and n-propanol can be explained by considering that large hydrophobic contributions are expected for these cosolvents, owing to the size of their apolar moieties, and that these contributions are larger for HbF than for HbA (see also fig. 7 below). Consistently, the experimental points to be fitted in order to determine the value of the parameter A in eq. 5 were chosen so as to give more weight to the cosolvents having smaller apolar moieties (formamide and methanol), since for these cosolvents smaller hydrophobic contributions are expected.

The broken straight line in fig. 6 has been obtained by a least-squares fit of four experimental points at low cosolvent concentration; these are three for formamide and one for methanol; the correlation coefficient is 0.99. The slope of this broken straight line (i.e., the value of the parameter A in eq. 5) is $4.14\ (\pm 0.31) \times 10^3$ kcal/mol and is approx. 25% greater than the analogous value of $3.35\ (\pm 0.11) \times 10^3$ kcal/mol reported for human adult and bovine hemoglobins.

We stress that, although the presence of deviations from the linear behavior at low concentrations of ethanol and n-propanol introduces some imprecision in the value of the parameter A relative to HbF, the conclusion that $A(\text{HbF}) > A(\text{HbA})$ is not to be questioned; indeed, if only the data points relative to formamide are used to determine the bulk electrostatic contributions to $\Delta\Delta G$ (in view of the rather large dipole moment and of the almost vanishing apolar moiety of the formamide molecule) an A value of 4.63×10^3 kcal/mol, i.e., greater than that reported above, is obtained. We, therefore, conservatively assume for HbF the A value to be $4.14\ (\pm 0.31) \times 10^3$ kcal/mol.

Having assumed that bulk electrostatic contributions can be calculated from eq. 5 throughout the whole cosolvent concentration range investigated, the non-bulk electrostatic (hydrophobic) contributions to $\Delta\Delta G$ due to the presence of cosolvents can be calculated as:

$$\Delta\Delta G_{\text{nbes}}(C) = \Delta\Delta G(C) - \Delta\Delta G_{\text{bes}}(C) \qquad (6)$$

These contributions are shown in fig. 7 as a function of cosolvent concentration. As can be seen from fig. 7, hydrophobic contributions are

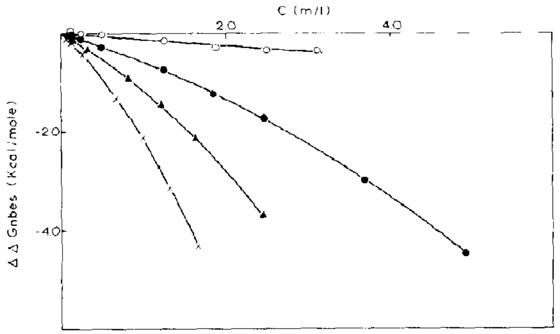

Fig. 7. $\Delta\Delta G_{\text{nbes}}$ as a function of cosolvent concentration. Symbols and experimental conditions as in fig. 2.

negative (i.e., in favour of the R conformation) and increase in absolute value with increasing cosolvent concentration; moreover, at constant concentration, they scale in the order formamide ≪ methanol < ethanol < *n*-propanol, i.e., with the size of the apolar part of the cosolvent molecules. This behavior is analogous to that already reported for human adult and bovine hemoglobins; therefore, also for HbF, we attribute the hydrophobic contributions to a smaller free energy needed, in the presence of apolar cosolvents, to expose to the solvent hydrophobic surfaces, following the T → R transition. It should also be noted that hydrophobic contributions are larger by about a factor of 2 in the case of HbF than of HbA.

4. Discussion

The experimental data shown in figs 6 and 7 indicate that, for HbF, both bulk electrostatic and hydrophobic contributions are larger than for HbA. This implies that in HbF at the T → R transition a greater number of favourable electrostatic interactions are broken and a larger hydrophobic surface is exposed to the solvent.

Consistently, the data reported in table 3 show that $L(\mathrm{HbF}) > L(\mathrm{HbA})$; in fact, we suggest that, in fetal hemoglobin, the T structure is more stabilized with respect to the R structure by an increased number of favourable electrostatic interactions and by the fact that a larger hydrophobic surface is exposed to the solvent following the T → R transition.

That $L(\mathrm{HbF}) > L(\mathrm{HbA})$ had already been suggested by Wind et al. [4]; these authors, however, did not propose any hypothesis on the physical origin of this effect. We believe that our data support the suggestion that a relevant contribution to the greater value of the allosteric constant relative to HbF (and therefore to its intrinsic lower oxygen affinity) arises from increased electrostatic interactions among charges on the protein surface and increased protein-solvent hydrophobic interactions.

The observation that $A(\mathrm{HbF}) > A(\mathrm{HbA})$ is consistent with the suggestion by Bonaventura and co-workers [5] that the T structure of HbF is more stable than that of HbA, since the former, due to the H21(143)His $\beta \rightarrow$ Ser γ substitution, has fewer repelling positive charges in the central cavity (it should be noted that the positive charges of H21(143)His β are absent in the R conformation of HbA, due to the Bohr effect). Indeed, the A value relative to HbA results from the positive contributions of Perutz's salt bridges [31] and from the negative contributions of the repelling charged groups in the central cavity that are, at least partially, exposed to the solvent. In deoxy HbF the negative contributions from the repulsion of the charged groups in the central cavity are diminished and this fact would explain the larger A value observed.

In order to propose a molecular mechanism able to rationalize the observation that hydrophobic contributions are larger for HbF than for HbA, we rely on the crystallographic study of the structure of human fetal deoxyhemoglobin by Frier and Perutz [6]. In deoxy-HbA the side chain of A4(7)Glu β forms a salt bridge with the N_ϵ of H10(132)Lys β of the same β-chain; this salt bridge has been found to be intact in the structure of oxyhemoglobin [36]. In the γ-chain the A4(7)Glu is substituted by Asp; since the aspartate side chain is shorter by 2.5 Å, formation of the above salt bridge in deoxy-HbF brings the A helix of the γ-chain closer to the molecular dyad axis, as indeed was found by Frier and Perutz [6]. We suggest that (in contrast to HbA) in the R conformation of HbF the salt bridge between A4(7)Asp γ and H10(132)Lys γ is lengthened or broken and that the two charged groups are at least partially exposed to the solvent; as a consequence the NA segment and the initial part of the A helix gain more conformational freedom and also become more exposed to the solvent. This would contribute to the greater A value observed for HbF and would explain why hydrophobic contributions to $\Delta\Delta G$ are, for HbF, larger than for HbA; the substitution NA3(3)Leu $\beta \rightarrow$ Phe γ in the terminal NA segment would also add to the larger hydrophobic effect observed in HbF. This molecular mechanism remains speculative; a direct test of its validity could be obtained from the solution of the structure of oxy-HbF.

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