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Stereochemistry of ATP and GTP Bound to Fish Haemoglobins

A Transferred Nuclear Overhauser Enhancement, ^{31}P-Nuclear Magnetic Resonance, Oxygen Equilibrium and Molecular Modelling Study

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This study was undertaken in order to test the models of ATP and GTP binding to carp deoxyhaemoglobin proposed by Perutz & Brunori (1982) and to find out why GTP is a more potent allosteric effector than ATP. We have determined the conformations of both nucleoside triphosphates by nuclear magnetic resonance studies and found them to be the same. The purines are in *anti* conformation about the glycosidic bond that links them to the ribose; the pentose ring is 3′-*endo*; the P–O5′–C5′–C4′ torsion angle lies in the *trans* domain (180° ± 20°); the P_α–O–P_β and P_β–O–P_γ angles are as in the free nucleotides, i.e. the trinucleotide chain is fully extended. Models having this conformation were fitted, first manually and then by energy refinement, to the effector site of an atomic model of human deoxyhaemoglobin in which the side-chains in the NA, EF and H segments had been replaced by those of carp. The results showed the location of the polar groups in carp haemoglobin to be such that $(PO_4)_\gamma$ can accept hydrogen bonds from Val NA1β_2 and from Arg H21β1, while $(PO_4)_\beta$ and $(PO_4)_\alpha$ can accept hydrogen bonds from Lys EF6β_1 and β_2. In ATP, the 6-amino group of the purine can donate a hydrogen bond to Glu NA2β_1. In GTP, the 2-amino group can donate a hydrogen bond to Glu NA2β_1; in addition, Val Na1β_1 can donate a hydrogen bond to O2′ of the ribose. This additional hydrogen bond may explain why in carp haemoglobin GTP is a stronger allosteric effector than ATP. We have found the influence of the two allosteric effectors on the oxygen affinity of trout IV haemoglobin to be the same, even though the only difference in the lining of

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the allosteric effector sites lies in the replacement of Glu Na2β in carp by Asp in trout IV haemoglobin. Model building then showed that formation of a hydrogen bond between Asp Na2β and the 2-amino group of guanine precludes formation of a hydrogen bond between Val NA1β and O2′ of the ribose or *vice versa*, which makes the number of hydrogen bonds formed between trout IV haemoglobin and GTP the same as those formed with ATP.

1. Introduction

Mammalian erythrocytes contain D-2,3-bisphosphoglycerate in about equimolar proportion to haemoglobin; this combines specifically with deoxyhaemoglobin in a cleft between the two β-chains. The cleft is lined with four pairs of cationic groups, which form a constellation of charges that is complementary to the anionic charges of DPG†. In oxyhaemoglobin, the cleft closes up. In consequence, DPG binds to deoxyhaemoglobin about a hundred times more strongly than to oxyhaemoglobin (Imai, 1982), and it acts as an allosteric effector that shifts the oxygen equilibrium curve to the right. Erythrocytes of teleost (bony) fish use adenosine triphosphate, guanosine triphosphate or inositol pentaphosphate and probably also lactate as allosteric effectors in place of DPG (Gillen & Riggs, 1977; Isaacks *et al.*, 1977). While in mammalian haemoglobins, DPG lowers the oxygen affinity more than ATP, the opposite holds in teleost fish (Gillen & Riggs, 1971). Moreover, GTP has been found to lower the oxygen affinity of carp haemoglobin twice as much as ATP (Weber & Lykkeboe, 1978). So far, no fish haemoglobin structure has been solved by X-ray analysis, so that the methods used to find the conformations and binding sites of DPG and IHP in human haemoglobin cannot be applied there (Arnone, 1972; Arnone & Perutz, 1974).

Mammalian haemoglobins whose oxygen affinity is regulated by DPG have a hydrogen donor side-chain in position NA2β (His, Gln or Asn) and they have His in position H21β. Teleost fish have either Glu or Asp in position NA2β and Arg at H21β. Substitution of those side-chains in the atomic model of human deoxyhaemoglobin produces a constellation of charged groups stereochemically complementary to strain-free ATP. The model suggests that, when ATP is bound, the carboxylate of either Glu or Asp NA2β_1 accepts a hydrogen bond from the N-6 amino group of adenine; the amino group of Val1β_2 and the guanidinium group of Arg H21β_2 each donate a hydrogen bond to $(PO_4)_\gamma$, while Lys EF6β_1 and β_2 may donate hydrogen bonds to $(PO_4)_\alpha$ and $(PO_4)_\beta$, thus neutralizing the four negative charges of ATP. In this model, the adenine is in the *anti* position relative to the ribose (Perutz & Brunori, 1982).

Since the model was first proposed, Braunitzer and his colleagues have determined the amino acid sequence of rhinoceros haemoglobin (Mazur *et al.*, 1982). Its allosteric effector site shows only a single substitution compared with that of human haemoglobin, His NA2$\beta \rightarrow$ Glu, yet ATP lowers its oxygen affinity more than DPG, and GTP lowers it more than ATP, just as in carp (R. Baumann, unpublished results). This observation supports the existence of a hydrogen bond

† Abbreviations used: DPG, D-2,3-bisphosphoglycerate; IHP, inositol hexaphosphate; NMR, nuclear magnetic resonance; D, deuterium; NOE, nuclear Overhauser effect; TRNOE, transferred NOE; p.p.m., parts per million.

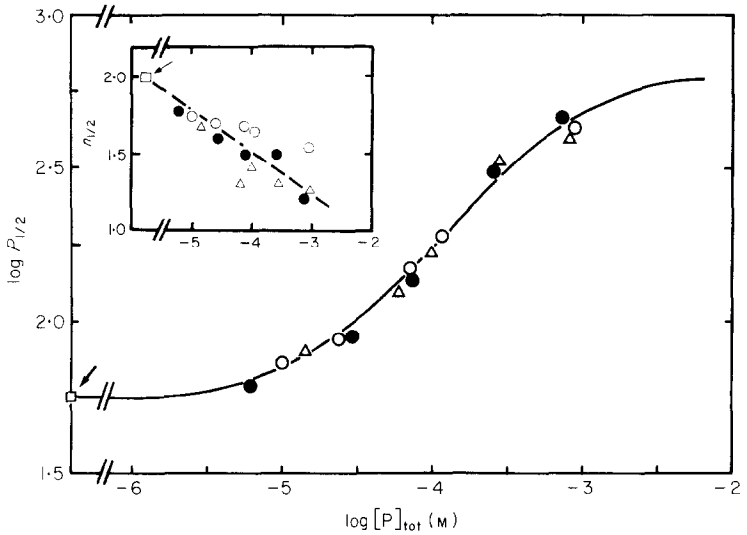

FIG. 1. Effect of the concentration of nucleoside triphosphates ($[P]_{tot}$) on the O_2 affinity of trout Hb IV at pH 7·0. Symbols: ○, ATP; ●, 8-Br-ATP; △, 8-Br-GTP; □, stripped. Conditions: [Hb], 3·5 mg/ml; bis-Tris, 0·05 M; and $[Cl^-]$, 0·02 M; temperature, 20°C. The inset shows the dependence of the Hill coefficient $n_{\frac{1}{2}}$ on organic phosphate concentration. $P_{\frac{1}{2}}$ is the partial pressure of oxygen at which the haemoglobin is half-saturated with oxygen.

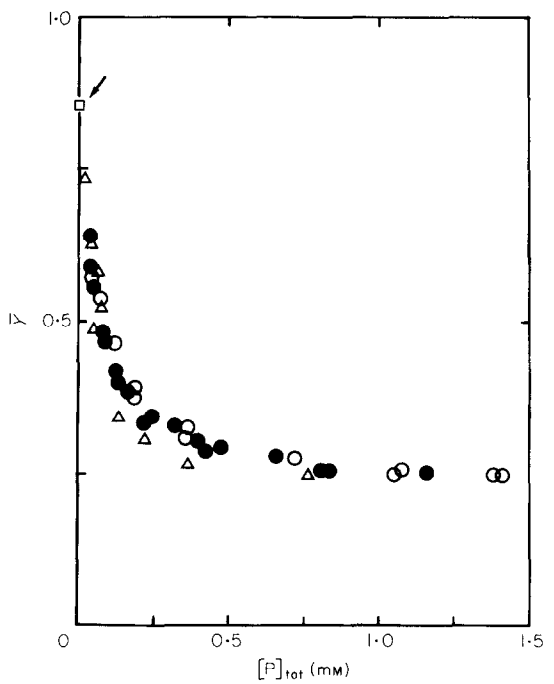

FIG. 2. Dependence of Y (fractional saturation with oxygen) in air as a function of increasing concentrations ($[P]_{tot}$) of nucleoside triphosphates (ATP, 8-Br-ATP and GTP; symbols as in Fig. 1). Conditions: [Hb], 3 to 5 mg/ml; bis-Tris buffer, 0·05 M, pH 7·0; $[Cl^-]$, 0·1 M; temperature, 20°C. The fractional O_2 saturation was calculated from visible absorption spectra.

between the purine and Glu NA2β, in fact, it can hardly be explained without it, but the stereochemistry of that bond in the GTP complex was not clear. A hydrogen bond between the 2-amino group of the guanine and Glu NA2β seemed possible only with the purine in the *syn* position, but there was no reason why this single hydrogen bond should be stronger than that formed by the 6-amino group of adenine, whence this structure failed to account for GTP being a stronger allosteric effector than ATP. A further doubt arose when it was pointed out to us that Perutz & Brunori's (1982) model could be tested by comparing the affinity of ATP for fish haemoglobin with that of 8-bromo-ATP, in which steric hindrance between the bromine and the ribose had been reported to lock the purine in the *syn* conformation (Bugg & Thewald, 1969). In that conformation, the hydrogen bond between the 8-amino group of adenine and Glu NA2β cannot be made in a stereochemically satisfactory way. In consequence, 8-bromo-ATP should be a weaker effector than ATP. However, when we compared the influence of ATP and 8-bromo-ATP on the oxygen affinity of trout IV haemoglobin we found them to be equal (Figs 1 and 2). We therefore concluded that Perutz & Brunori's model must be wrong, even though it looked stereochemically very attractive. These apparent contradictions led us to probe the conformations of ATP and GTP bound to carp deoxyhaemoglobin by nuclear magnetic resonance spectroscopy. In particular, we have used the proton–proton transferred nuclear Overhauser effect (Clore & Gronenborn, 1982,1983; Clore *et al.*, 1984; Gronenborn & Clore, 1982*a*,*b*; Gronenborn *et al.*, 1984) to determine the angle of rotation about the glycosidic bond and the pucker of the ribose, and employed ^{31}P-NMR spectroscopy to determine the angle of rotation about the C5′–O5′ bond and to obtain qualitative information on the environment of the phosphorus atoms.

2. Experimental Procedures

(a) *Oxygen equilibrium measurements*

Component IV of trout haemoglobin (trout Hb IV) was purified from trout blood as described by Binotti *et al.* (1971). Oxygen equilibrium measurements were obtained by the method of Rossi Fanelli & Antonini (1958).

(b) *NMR spectroscopy*

Carp haemoglobin, prepared as described by Condo *et al.* (1981) was extensively dialysed against D_2O. Samples for NMR were prepared in a nitrogen-filled glove box and NMR tubes were sealed with beeswax. Samples for ^{1}H-NMR were made up in 99·6% D_2O and contained 0·5 mM-carp deoxyhaemoglobin (in tetramer), 8·3 mM-sodium dithionite, 5·5 mM-nucleoside triphosphate, 288 mM-KCl, 28·8 mM-potassium phosphate, pH 6·7 (meter reading uncorrected for the isotope effect on the glass electrode) and 0·8 mM-EDTA. Samples for ^{31}P-NMR were made up in 90% H_2O/10% D_2O and contained 1·4 mM-carp deoxyhaemoglobin (in tetramer), 24 mM-sodium dithionite, 1·4 or 2·8 mM-nucleoside triphosphate, 100 mM-Tris·HCl, (pH 6·9) and 1 mM-EDTA. ^{1}H chemical shifts were measured with respect to 2,2 dimethylsilapentane-5-sulphonate as an external standard. ^{31}P chemical shifts were measured relative to 85% orthophosphoric acid as an external standard.

^{1}H-NMR spectra were obtained at 270 MHz using a Bruker WH-270 spectrometer: 300 transients, obtained by quadrature detection with 4096 data points and a spectral width of 4·2 kHz, were averaged for each spectrum. Prior to Fourier transformation, the free

induction decay was multiplied by an exponential equivalent to a line broadening of 2 Hz. The pulse sequence used in the time-dependent TRNOE measurements was $(t_1-t_2-\pi/2-AT-t_3)_n$, where the selective irradiation at a chosen frequency is applied during the time interval t_1 (0·002 to 0·6 s), t_2 is a short delay (2 ms) to allow for electronic recovery after the removal of the selective irradiation, AT is the acquisition time (0·437 s), and t_3 is a relaxation delay (3 s) to allow for complete recovery of magnetization of all protons to their equilibrium values prior to perturbation by the radiofrequency field. The irradiation power used was sufficient to be in the high power limit, so that saturation is effectively instantaneous whilst selectivity is preserved so that only a single average resonance at a time was saturated (Dobson *et al.*, 1982; Clore & Gronenborn, 1983; Gronenborn *et al.*, 1984). As an initial procedure, the spectral region from 3·4 to 7·2 p.p.m. was systematically irradiated at 20 Hz (0·074 p.p.m.) intervals using a 0·4 s pre-saturation pulse. This region covers all the sugar resonances of the nucleoside triphosphates and the α and β CH protons of the protein. In this procedure, the selectivity of the TRNOEs is maintained since, in general, the extent of spin-diffusion from indirect cross-relaxation *via* protons of the protein is approximately independent of the irradiation frequency, providing this is placed within the protein resonance envelope (Clore & Gronenborn, 1982,1983). All the observed TRNOE effects were maximal and centred at the positions of the averaged ligand resonances (note free and bound nucleoside triphosphate are in fast exchange on the chemical shift scale), indicating that the TRNOEs arise as a result of direct cross-relaxation between bound ligand protons (Clore & Gronenborn, 1982,1983; Gronenborn & Clore, 1982*a*,*b*; Gronenborn *et al.*, 1984).

^{31}P-NMR spectra were recorded at 81 MHz on a Bruker WM200 spectrometer with an acquisition time of 1·024 s (8192 data points and a spectral width of 8 kHz), a pulse-width of 45° and an interpulse relaxation delay of 1 s: 6000 transients were averaged for each spectrum. Prior to Fourier transformation, the free induction decay was multiplied by an exponential equivalent to a line broadening of 2 Hz.

3. Results and Discussion

(a) *NMR spectroscopy*

Clore & Gronenborn (1983) have described the theory of the time-dependent TRNOE for an exchanging system containing multiple spins as applied to the conformations of ligands bound to proteins. We found that the chemical exchange of nucleoside triphosphates with carp deoxyhaemoglobin is fast on the chemical shift scale, so that only a single set of exchange-broadened averaged ligand resonances is observed, and that *no* NOEs are observed for the free ligands in the absence of protein at irradiation times of less than one second. Consequently, the initial slopes of the time-dependent TRNOE of the averaged ligand resonance i following irradiation of the averaged ligand resonance j is simply given by $-(1-a)\sigma_{ij}^{BB}$, where a is the mole fraction of free ligand and σ_{ij}^{BB} is the cross-relaxation rate between the bound ligand protons i_B and j_B. Note that the cross-relaxation rates in the ligand–protein complex for which $\omega\tau_c \gg 1$ (where ω is the Larmor frequency and τ_c the correlation time) are of opposite sign to those in the free ligand for which $\omega\tau_c \ll 1$. As a result, the sign of the TRNOE is negative as opposed to the positive steady-state NOE for the free ligand. The cross-relaxation rate σ_{ij}^{BB} is proportional to $\langle(r_{ij}^{BB})^{-6}\rangle$, where r_{ij}^{BB} is the distance between protons i_B and j_B is the ligand–protein complex. This relationship has two consequences: (1) distance ratios between pairs of bound ligand protons can be determined directly and with ease from the initial slopes of the time-dependent TRNOEs; and (2) the value of the cross-relaxation rate decreases rapidly as the interproton

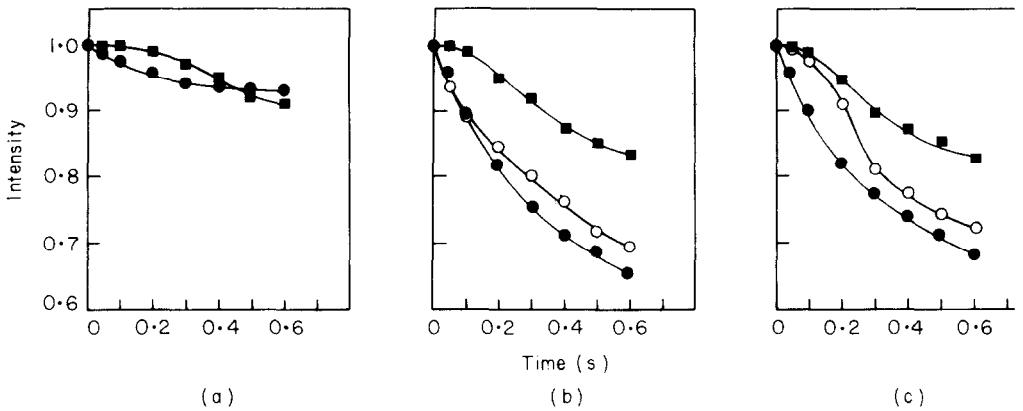

FIG. 3. Time dependence of TRNOEs observed on the averaged H8, H2 and H1′ resonances of ATP (5·5 mM) following irradiation of (a) the averaged H1′, (b) H2′ and (c) H3′ resonances of ATP in the presence of carp deoxyhaemoglobin (0·5 mM in tetramer) with a ratio of free to bound ligand of 10. Symbols: ●, H8 resonance; ■, H2 resonance; ○, H1′ resonance. The sample temperature was 0°C. The experimental conditions were as given in Experimental Procedures. (Note that the free and bound states of ATP are in fast exchange on the chemical shift scale, so that there is only a single set of averaged ligand resonances whose positions, under these experimental conditions, are approximately the same as those of the corresponding resonances in free ATP.)

distance increases and becomes negligible for $r_{ij}^{BB} \gtrsim 4$ Å. Thus, for distances less than 4 Å, the intensity of the averaged ligand resonance on which the TRNOE is observed initially decreases with time, while for distances greater than 4 Å it exhibits a lag phase.

Figure 3 illustrates a set of time-dependent TRNOE experiments on ATP bound to carp deoxyhaemoglobin in the presence of a tenfold excess of free ATP. Direct TRNOEs are observed on the H8 resonance following irradiation of the H1′, H2′ and H3′ resonances with initial slopes of 0·2, 1·08 and 1·05 s^{-1}, respectively, and on the H1′ resonance following irradiation of the H2′ resonance (initial slope of 1·10 s^{-1}). In contrast, lag phases, characteristic of indirect TRNOE effects, are observed on the H2 resonance following irradiation of the H1′, H2′ and H3′ resonances, and on the H1′ resonance following irradiation of the H3′ resonance. The same pattern of TRNOEs is observed on the resonances of GTP bound to carp deoxyhaemoglobin.

Table 1 gives the complete set of cross-relaxation rates between the protons of bound ATP and GTP together with the distance ratios calculated from them. For both ATP and GTP, the TRNOE data indicate that the distances $r_{H8-H2'}^{BB}$, $r_{H8-H3'}^{BB}$ and $r_{H1'-H2'}^{BB}$ are approximately equal, that the distance $r_{H8-H1'}^{BB}$ is approximately 25 to 30% longer than the $r_{H8-H2'}^{BB}$ distance, and that the distances $r_{H8-H5'}^{BB}$ and $r_{H8-H5''}^{BB}$ are both greater than 4 Å. These distance ratios indicate a high *anti* conformation about the glycoside bond [χ(O4′–C1′–N9–C4) ~ 290 ± 20°] and a pure 3′-endo sugar pucker for both ATP and GTP†. The TRNOE data, however, do

† The glycosidic bond torsion angle χ is defined by the atoms O4′–C1′–N9–C4 with zero at the *cis* position and positive angles by clockwise rotation of the further pairs of atoms; this is the new IUPAC convention, and the angle is 180° different from the angle defined by the earlier convention; i.e. $\chi_{new} = \chi_{old} - 180$; i.e. $-70°(=290°)$ $\chi_{new} = 110°\chi_{old}$.

TABLE 1

Cross-relaxation rates for ATP and GTP bound to carp deoxyhaemoglobin determined from time-dependent TRNOE measurements at 0°C together with the interproton distance ratios calculated from them

Irradiated resonance	Observed resonance	ATP		GTP	
		σ_{ij}^{BB} (s^{-1})	$r_{ij}^{BB}/r_{H8-H2'}^{BB}$	σ_{ij}^{BB} (s^{-1})	$r_{ij}^{BB}/r_{H8-H2'}^{BB}$
H1′	H8	2·0	1·32	2·0	1·23
H2′	H8	10·8	1·00	7·0	1·00
H3′	H8	10·5	1·00	8·8	0·96
H5′/H5″†	H8	Lag‡		Lag‡	
H2′	H1′	11·1	1·00	6·0	1·03
H3′	H1′	Lag‡		Lag‡	
H5′/H5″†	H1′	Lag‡		Lag‡	
H1′	H2	Lag‡			
H2′	H2	Lag‡			
H3′	H2	Lag‡			
H5′/H5″†	H2	Lag‡			

The relative errors in the values of the cross-relaxation rates are $\lesssim \pm 0{\cdot}10$. The interproton distance ratios are calculated from the equation $r_{ij}^{BB}/r_{kl}^{BB} = (\sigma_{kl}^{BB}\sigma_{ij}^{BB})^{1/6}$ on the assumption of a single correlation time for all interproton distance vectors. The errors in the values of the distance ratios are $\lesssim \pm 0{\cdot}03$.

† The H5′ and H5″ resonances are superimposed.

‡ The presence of a lag phase indicates that $r_{ij}^{BB} \gtrsim 4$ Å.

not define the O5′–C5′–C4′–C3′ torsion angle, aince the *anti* glycoside bond and pure 3′-endo sugar pucker render the distances between the H8 proton and the H5′ and H5″ protons $\gtrsim 4$ Å for all rotations about the C4′–C5′ bond.

The conformation about the O5′–C5′ bond can be deduced from ^{31}P-NMR measurements. Figure 4 shows the ^{1}H-decoupled and coupled ^{31}P-NMR spectra of a 1 : 1 complex of ATP with carp deoxyhaemoglobin. The increase in line-width of the P_α resonance in the coupled spectrum relative to that in the decoupled spectrum is only ~ 5 Hz and reflects the sum of the two three-bond ^{1}H-^{31}P coupling constants, $^{3}J_{P_\alpha}$–*H5′* and $^{3}J_{P_\alpha}$–H5″. The same result is found in the case of GTP. ^{31}P-NMR studies on cyclic nucleotides have shown that $J_{trans} = 20{\cdot}9$ Hz and $J_{gauche} = 1{\cdot}8$ Hz (Blackburn *et al.*, 1973). Thus the ^{31}P-NMR data indicate that the conformation about the C5′–O5′ bond of ATP and GTP bound to carp deoxyhaemoglobin is such that the P_α atom is *gauche* to both the H5′ and H5″ protons; that is to say, the P–O5′–C5′–C4′ torsion angle lies in the *trans* domain ($180 \pm 20°$).

The ^{31}P-NMR data also provide qualitative information on the P_α–O–P_β and P_β–O–P_γ angles. Both the $^{2}J_{P_\alpha P_\beta}$ and $^{2}J_{P_\beta P_\gamma}$ coupling constants are the same as in the free state (19·5 Hz), so that the P–O–P angles are probably unchanged upon binding to carp deoxyhaemoglobin.

Turning to the ^{31}P chemical shifts, we note that the P_γ resonance undergoes the most substantial shift upon binding to carp deoxyhaemoglobin (+1·28 and +1·73 p.p.m. for ATP and GTP, respectively), followed by the P_β resonance (+0·51 and +0·80 p.p.m. for ATP and GTP, respectively), and finally the P_α resonance, which barely shifts at all (−0·22 and −0·25 p.p.m. for ATP and GTP,

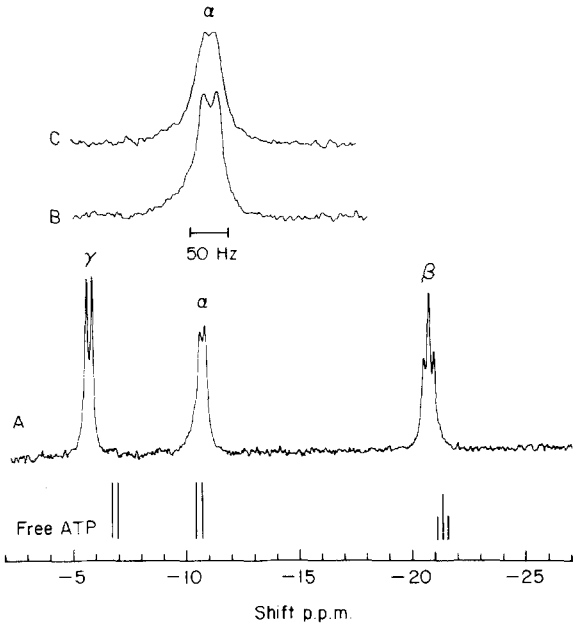

FIG. 4. ^{31}P-NMR spectra of ATP in a 1 : 1 complex of carp deoxyhaemoglobin with ATP. Trace A, ^{1}H-decoupled spectrum; trace B, expansion of the P_α resonance with ^{1}H-decoupling; trace C, expansion of the P_α resonance without ^{1}H-decoupling. A stick diagram of the ^{1}H-decoupled spectrum of free ATP is also shown. The sample temperature was 20°C. The experimental conditions were as given in Experimental Procedures.

respectively). The large downfield shifts experienced by the P_γ and P_β resonances can be explained by the proximity of positively charged amino acid side-chains in the nucleoside triphosphate binding site of carp deoxyhaemoglobin, which polarize electrons surrounding the P_α and P_β atoms, thereby changing their induced magnetization (i.e. a linear electric field effect as has been proposed for other nucleotide–protein complexes; Feeney *et al.*, 1975).

(b) *Construction of models*

On the basis of the conformations of ATP and GTP determined by NMR, we have built molecular models of the interactions between the nucleoside triphosphates and deoxyhaemoglobin. Initially, we used the Kendrew model of human deoxyhaemoglobin derived from Fermi's (1975) refinement at 2·5 Å resolution, in which we merely replaced the side-chain of His NA2 by Glu and that of His H21 by Arg. When satisfactory models had been constructed, their atomic co-ordinates were measured. These rough co-ordinates were then subjected to several cycles of energy refinement by the method of Jack & Levitt (1978); the conformations of the nucleotides were held rigid as determined by NMR, and their positions in the effector site were allowed to vary. To make the calculations more realistic, all the side-chains in the NA, EF and H segments of human haemoglobin were replaced by those of carp.

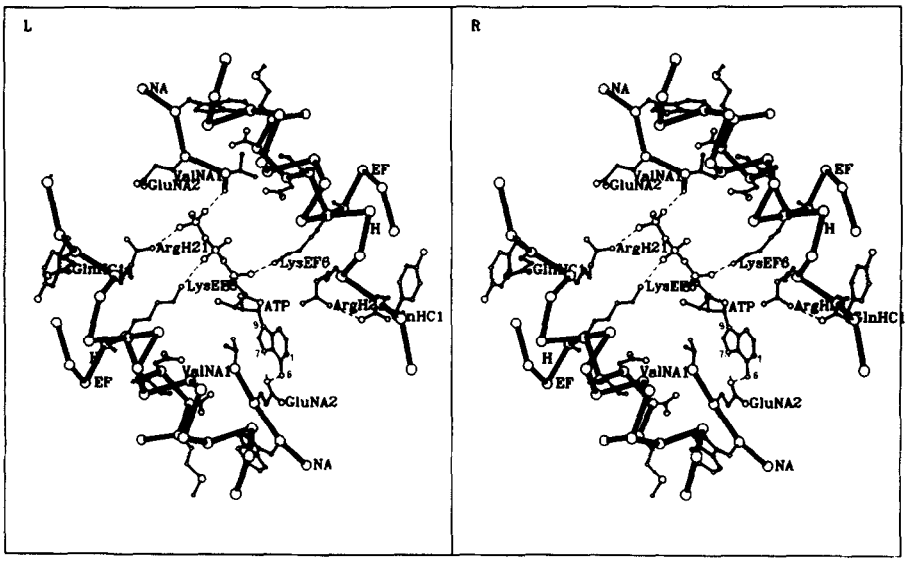

FIG. 5. Proposed binding of ATP to carp deoxyhaemoglobin.

The structure of the ATP complex determined in this manner was the same as that proposed by Perutz & Brunori (1982), and is shown in Figure 5. The 6-amino group of adenine donates a hydrogen bond to Glu NA2β_1; $P_\gamma O_4$ accepts hydrogen bonds from Val NA1β_2 and Arg H21β_1; $P_\alpha O_4$ and $P_\beta O_4$ accept hydrogen bonds from the two lysine EF6β residues. In GTP, the amino group lies in position 2 of the purine. If GTP is inserted into the effector site in the same orientation as ATP, its amino group faces away from Glu NA2β_1. To make the vital hydrogen bond, the entire nucleotide had to be rotated by about 180° about its long axis. As soon as this was done, all the bonds fell into place (Fig. 6). The 2-amino group bonds to Glu NA2β_1 with the purine almost coplanar with the carboxylate. The phosphates accept hydrogen bonds from Val NA1β_2, Arg H21β_1 and the two Lys EF6β residues as in the ATP complex. The inversion of the nucleotide allows the formation of one additional hydrogen bond that is not possible in the ATP complex. This is from Val NA1β_2 to the O2′ of the ribose. Is this bond sufficient to account for the stronger effect on the oxygen affinity of GTP as compared to ATP? The free energy $-E$ contributed to the stability of the T structure by one additional bond can be calculated from its effect on $P_{\frac{1}{2}}$, the partial pressure of oxygen at which the haemoglobin solution is half saturated with oxygen, from the equations:

$$\Delta \log P_{\frac{1}{2}} = 1/4\Delta \log L \tag{1}$$

$$-E = RT2{\cdot}303\Delta \log L, \tag{2}$$

where L is the allosteric constant. The twofold difference between the effects of ATP and GTP on the $P_{\frac{1}{2}}$ value of carp haemoglobin gives $-E = 1{\cdot}6$ kcal (1 kcal = 4·184 kJ), which is consistent with the contribution of one additional hydrogen bond.

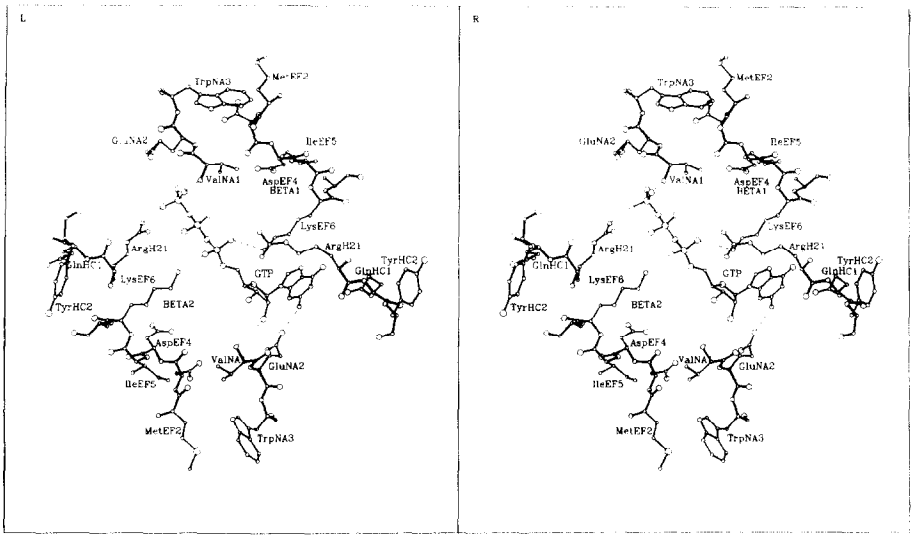

FIG. 6. Proposed binding of GTP to carp deoxyhaemoglobin. Note that O2′ and O3′ point up in this structure, towards Val NA1β_2, while in the ATP structure they point down, away from Val NA1β_2.

Expecting trout IV haemoglobin to exhibit the same behaviour as carp haemoglobin, we used the former rather than the latter because it was readily available and had long been studied in our Rome laboratory. While GTP raises the $P_{\frac{1}{2}}$ value of carp and rhinoceros haemoglobins more than ATP, we were surprised to find that the two effectors raised the $P_{\frac{1}{2}}$ value of trout IV haemoglobin equally (Figs 1 and 2). The effector site of trout IV haemoglobin differs from that of carp only by the substitution of Asp for Glu in position NA2β. Model building suggests that formation of a hydrogen bond between the 2-amino group and Asp NA2β_1 removes the O2′ of the ribose from hydrogen-bonding range with Val NA1β or *vice versa*. This substitution may thus inhibit the formation of the additional hydrogen bond that makes GTP the stronger effector in carp haemoglobin.

4. Concluding Remarks

We built our models by taking the co-ordinates of human deoxyhaemoglobin given by Fermi *et al.* (1984) and merely replacing the side-chains of the NA, EF and H segments by those in carp. Our structure therefore rests on the assumption that the tertiary and quaternary structures of human and carp haemoglobins are the same. When this proposal was made by Perutz (1983), it was met with disbelief among biologists and others, because the number of amino acid substitutions between human and fish haemoglobins is about 140, or half the total. However, it is important to realise that only a few of these substitutions lie at haem contacts or at functionally important contacts between the subunits. Of the residues in contact with the haems in human haemoglobin listed by Fermi & Perutz (1981), 16 out of 20 are identical in the α-chains and 14 out of 20 in the

β-chains. At the $\alpha_1\beta_2$ contact, which forms the switch between the deoxy and oxy structures, 27 out of 32 residues are identical in the two species. These homologous residues would be stereochemical misfits unless the tertiary and quaternary structures superimposed with a standard deviation of less than 1 Å.

The α-chain of carp haemoglobin contains one extra residue compared to human, but this lies in the external and non-helical CD segment where it can be accommodated by only local modifications of structure. It has been argued that the paramagnetically shifted haem proton resonances of carp deoxyhaemoglobin are diffuse and bear no resemblance to those of human haemoglobin, which suggests at first sight that the two proteins have different structures, but La Mar and his colleagues have discovered the explanation to be different. It appears that in both species bound haems exchange; on rebinding to the globin they may come to lie either side up. In human haemoglobin, over 90% are one side up, which is the dominant structure found in X-ray and NMR studies. In carp, on the other hand, the two structures are about equally populated, so that the vinyl and methyl protons of the porphyrin see two different environments. This multiplicity broadens their resonances (La Mar *et al.*, 1984).

How are the equal effects of ATP and 8-bromo-ATP on the $P_{\frac{1}{2}}$ value of trout haemoglobin to be reconciled with our structure? This apparent contradiction has been resolved by the discovery by Abdallah *et al.* (1975) that steric hindrance between the bromine and the ribose, which seemed to force the purine into the *syn* conformation in crystals of 8-bromo-ATP, is relieved when the nucleotide is bound to alcohol dehydrogenase by switching the ribose from the 3′ to the 2′-*endo* conformation, thus allowing the purine to take up the *anti* conformation, as in ATP.

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