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Structure of hemoglobins Zürich [His E7(63)β→Arg] and Sydney [Val E11(67)β→Ala] and role of the distal residues in ligand binding

(abnormal hemoglobins/x-ray analysis/molecular structure/infrared absorption)

P. W. TUCKER*†, S. E. V. PHILLIPS*, M. F. PERUTZ*‡, R. HOUTCHENS§, AND W. S. CAUGHEY§

* Medical Research Council Laboratory of Molecular Biology, Cambridge CB2 2QH, England; and § Department of Biochemistry, Colorado State University, Fort Collins, Colorado 80523

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ABSTRACT **In hemoglobin Zürich the side chain of the distal arginine attaches itself to the propionate of the heme, leaving the heme pocket wide open, allowing sulfanilamides easy access to the iron, and doubling the partition coefficient between CO and O_2. The replacement of the distal valine by alanine in hemoglobin Sydney leaves a large gap inside the heme pocket, which is partly filled by a water molecule bonded to the distal histidine. Hemoglobin Sydney has the same partition coefficient between CO and O_2 as hemoglobin A. Replacement of the distal histidine increases the stretching frequency of CO linked to the β heme by 6 cm^{-1}, but replacement of the distal valine increases it by only 3 cm^{-1}. Replacement of the distal histidine leaves the O–O stretching frequency unchanged.**

The iron atom in hemoglobin (Hb) is bonded to a histidine on the proximal side of the heme; on the distal side of the heme it is faced by another histidine (E7) and a valine (E11). Two atoms of the histidine, C_ϵ and N_ϵ, are in van der Waals contact with the porphyrin in both deoxyHb and oxyHb, and N_ϵ is also in contact with heme ligands. The histidine side chain also acts as a gate to the heme pocket, not allowing ligands to enter or leave unless it swings out of the way. The role of Val E11 is more selective: its methyl γ_2 is in van der Waals contact with the porphyrin only in the β subunit of deoxyHb, where it overlaps the van der Waals radii of heme ligands, so that the heme pocket must widen before ligands can bind; this restriction either does not apply, or applies only to a much smaller extent, to the α subunit. There, on the other hand, access to the ligand site is blocked by a water molecule hydrogen bonded to His E7 (1–4). What role do these distal residues play in ligand binding and cooperativity? We have approached this question by x-ray crystallographic, infrared absorption, and chemical studies of two abnormal Hbs in which either the histidine or the valine has been replaced by another residue, leaving behind an empty space in the heme pocket.

Hb Zürich [His E7(63)β→Arg] causes hemolytic inclusion body anaemia on treatment of it heterozygous carriers with sulfanilamides (5–7); it has an abnormally high oxygen affinity, low cooperativity, and normal Bohr effect (8); *in vitro*, but normally not *in vivo*, its β hemes are autoxidized more easily than those of Hb A (9). CO is displaced by oxygen more slowly than in Hb A (10). Perutz and Lehmann pointed out that the side chain of Arg E7 could not be accommodated in the heme pocket, but would protrude at the surface, leaving a large cavity by the ligand site of the iron (11).

Hb Sydney [Val E11(67)β→Ala] causes hemolytic anemia in heterozygous carriers; it is easily autoxidized, and is unstable, losing heme on heating to 50° (12). Because replacement of the distal valine by alanine removes an obstruction to ligand binding, one would have expected the oxygen affinity of Hb Sydney to be abnormally high, but, in fact, Lehmann and his colleagues found its mean affinity (p_{50}) to be normal. The oxygen equilibrium curve of a mixture of Hb A and Hb Sydney crosses that of Hb A, showing that the mixture has an abnormally high affinity at low and abnormally low affinity at high partial pressures of O_2 (13). This unexpected behavior made us suspect that the vacant space left by the removal of the methyl groups of the valine might be filled by a water molecule bonded to the distal histidine.

RESULTS

The difference electron density map of Hb Zürich shows a large negative peak covering part of the imidazole of His E7 and a dominant positive peak flanking the propionate side chain IV of the heme (Fig. 1). This peak clearly represents the side chain of Arg E7. There is also a negative peak by the side chain of Phe CD4, suggesting that it has been pushed upwards, and there are some negative peaks to the left of the bottom edge of the heme, suggesting that this edge has moved to the distal side, so that the heme as seen in the figure has turned counterclockwise. To determine the position of the arginine side chain, we fitted a model of it and of the heme to the difference density in a miniature Richards box (20). We kept the α and β carbons as in the structure of HbCO A (J. M. Baldwin, personal communication), because no difference density is associated with them, and fitted the rest of the side chain to the difference peak. We then measured the peak's coordinates and matched them to the remainder of the HbCO A structure. This placed one of the guanidinium nitrogens within 2.9 Å of one of the propionate oxygens, suggesting that the two groups are linked by a salt bridge (Fig. 2). There may also be a very weak hydrogen bond to Ser CD3. We could find no difference density on the heme-linked CO, but a slight change in its angle of tilt would not be detectable at our resolution. Note that the removal of His E7 not only leaves an empty space in the heme pocket, but also leaves the entrance to the pocket wide open. We next built a model of sulfanilamide and found that it can enter the heme pocket without distortion of the protein. Its sulfonamide could form a hydrogen bond with the heme propionate III, the one that does not interact with Arg E7; its amino group then comes to lie opposite the iron atom.

The electronic absorption spectra of Hb Zürich closely resemble those of Hb A. OxyHb Zürich has its Soret band red-shifted by ~1 nm; its α and β bands are indistinguishable from those of Hb A. DeoxyHb Zürich has its Soret band blue-shifted by ~2 nm, and the shoulder at 406 nm is more pronounced than in Hb A. The maximum of the visible band is in the same position as in Hb A, but a new shoulder at 574 nm either replaces or obscures the normal one at 588 nm. The CO stretching fre-

† Present address: Laboratory of Genetics, 406 Genetics Building, University of Wisconsin, Madison, WI 53706.
‡ To whom reprint requests should be addressed.

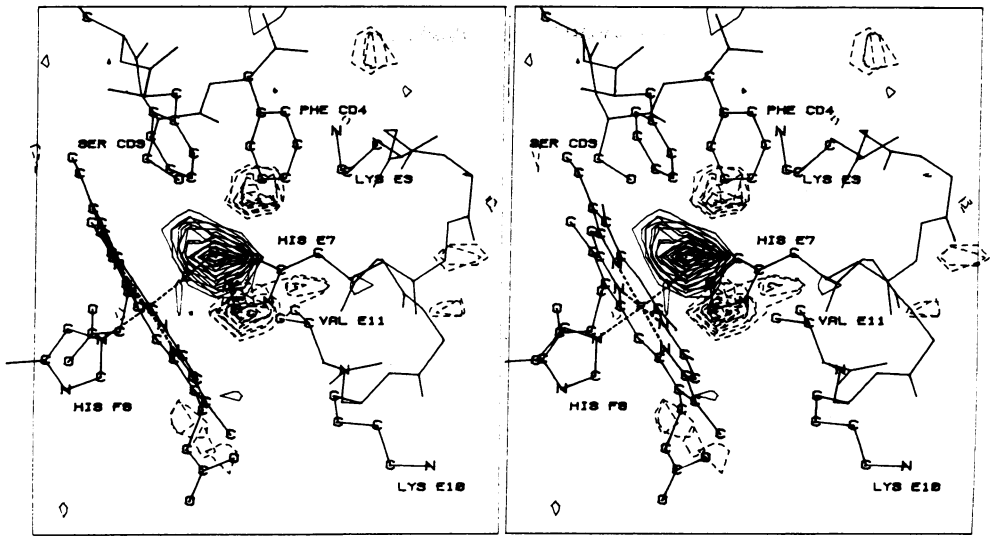

FIG. 1. Difference Fourier synthesis of HbCO Zürich minus HbCO A at 2.76-Å resolution superimposed on the structure of HbCO A. Section intervals are 0.75 Å, contour intervals are 0.025 electrons per Å^3 (zero level omitted), and negative contours are broken. Shown are sections +17 to +37, cut perpendicular to the crystallographic *c* axis; i.e., the β heme ligand pocket is seen from the "bottom" according to the conventional view in figures 3b and 17 of ref. 14. Crystals of HbCO Zürich grown by a vapor diffusion modification of the method described for crystallizing HbCO A (15) gave small tetragonal bipyramids isomorphous with HbCO A (16). $a = b = 53.7$ Å, $c = 193$ Å; space group $P4_12_12$ with one $\alpha\beta$ dimer in the asymmetric unit. X-ray intensities were recorded on an Arndt–Wonacott rotation camera (17) using graphite monochromatized Cu Kα radiation. A complete set of reflections within a limiting sphere of 2.76 Å was obtained from one crystal at 20–25° without appreciable degradation. The intensities were measured and processed (18) to give 7500 unique reflections with an overall film scaling standard deviation in structure amplitude *F* of 8.6%. A difference Fourier synthesis was calculated using ($|F_{CO\ Zürich}| - |F_{CO\ A}|$) as coefficients together with the phase angles of HbCO A derived from J. M. Baldwin's recent real space and energy-refined structure (unpublished). The mean isomorphous difference was 9.7% of the mean $|F_{CO\ A}|$ and the overall root-mean-square difference density was 0.038 electron per Å^3.

quency in the β subunit of Hb Zürich is raised from 1952 to 1958 cm^{-1} without significant change in band width (10).

Despite the mixed crystals used for the x-ray analysis, the difference map of deoxyHb Sydney and A is quite clear. The γ carbons of the replaced valine are covered by two well-resolved lobes of negative density (Fig. 3). The only other prominent feature is a large positive peak extending from N_ϵ of His E7 towards the center of the heme pocket; this is most readily interpreted as a water molecule hydrogen bonded to His E7 and filling part of the gap left by the replacement of Val E11 by Ala. One naturally suspects that its presence might signify the oxidation of the heme iron and formation of aquometHb, but this is unlikely for three reasons: the α hemes are entirely clear of difference density; the centroid of the difference peak lies 3.4 Å from the iron atom, compared to a separation of only 2.1 Å in methemoglobin; absorption spectra of crystals measured before and after exposure to x-rays showed no evidence of oxidation. The peak seems to represent a water molecule in contact with the porphyrin but not the iron atom, as in the α subunits of deoxyHb A and in deoxymyoglobin (3, 22). We next transformed the coordinates of the α and β hemes to an identical orientation and superimposed them. Fig. 4 shows that the water molecules in the two heme pockets occupy similar positions; the N_ϵ–H_2O distance is too short for a hydrogen bond (2.2 Å), but this could be relieved by turning the imidazole about the C_β—C_γ bond as indicated in the figure. The rotation would make the $N_{porphyrin}$–H_2O–N_ϵ angle more nearly tetrahedral and would explain why the positive peak contains 9% more density than it should if it represents only a water molecule, compared to the two negative peaks representing methyls. The small negative peak near N_ϵ also favors such a rotation. Fig. 4 also shows that C_β of Ala E11β occupies roughly the same position relative to the heme as does $C_{\gamma 2}$ of Val E11α, so that the replacement in Hb Sydney makes the abnormal β heme pocket somewhat similar to that of the normal α heme pocket. The valine methyls are further from His E7 in the α than in the β subunits of Hb A, so that they allow space for a water molecule bound to the histidine in the former but

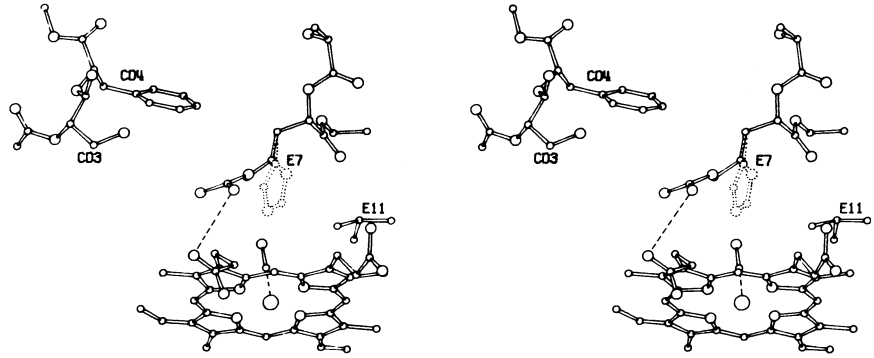

FIG. 2. Orientation of Arg E7 in the β heme ligand pocket of HbCO Zürich. The position of the replaced distal His E7 is denoted with dotted lines. The view is similar to that of Fig. 1.

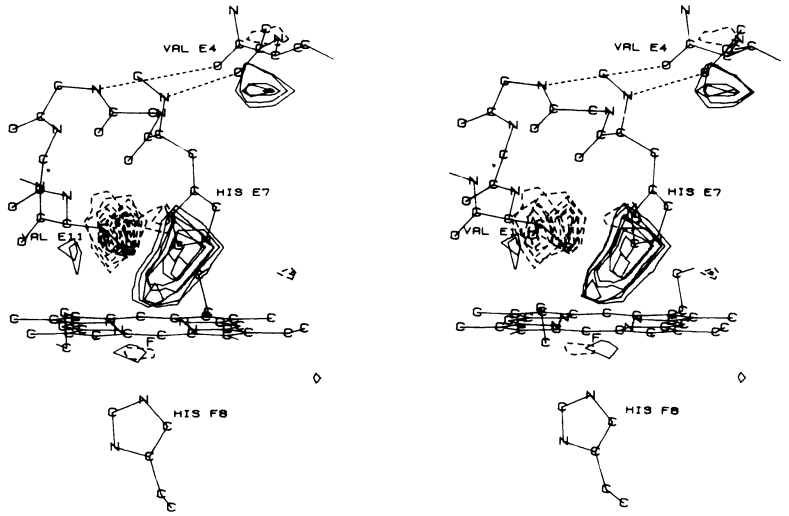

FIG. 3. Difference Fourier synthesis of deoxyHb Sydney minus deoxyHb A at 2.73-Å resolution superimposed on the refined atomic positions of the β heme and associated residues of deoxyHb A. Section intervals are 0.75 Å and each section is contoured at 0.018 electron per Å with zero levels omitted. Negative contours are broken lines. The figure shows sections −6 to +6 cut approximately perpendicular to the β heme plane. The view is from the top of the molecule down, according to the convention (figures 3b and 17) of ref. 14. Hb Sydney cannot be separated from Hb A. A mixture of the two deoxyHbs was crystallized (15), giving crystals isomorphous with Hb A, with a = 63.2 Å, b = 83.6 Å, c = 53.8 Å, β = 99.34°; space group $P2_1$ with one tetramer in the asymmetric unit. Reflections within a limiting sphere of 2.73 $Å^{-1}$ were measured on a diffractometer (21) to give 13,764 independent reflections with an overall standard deviation in $|F|$ of 4.1%; these were matched with the observed amplitudes of deoxyHb A (19). A difference Fourier synthesis was calculated using ($|F_{\text{Sydney + A}} - F_A|$) as coefficients together with the phase angles calculated from the real space refined structure of deoxyHb A (3). The mean isomorphous difference was 5.7% of the mean $|F_A|$ and the overall root-mean-square difference density was 0.029 electron per $Å^3$, much smaller than for Hb Zürich.

not in the latter. The infrared spectra of CO-saturated red cells from the same patient as those used for crystallization showed only the normal CO stretching frequency μ_{CO} at 1951 cm^{-1}, but its half-band-width is greater by 0.5 ± 0.1 cm^{-1}. Difference spectra of the mixture of HbCO Sydney and A minus HbCO A suggest that μ_{CO} for the abnormal β subunit is near 1955 cm^{-1} compared to 1952 cm^{-1} for the normal β subunit. The O–O stretching band of Hb Zürich has the same frequency (1106–1107 cm^{-1}), shape, and intensity as that of Hb A.

The partition coefficient between O_2 and CO is defined as $M = (pO_2[\text{HbCO}])/(p\text{CO}[\text{HbO}_2])$, in which p is pressure. For Hb A we found M = 250 (Fig. 5). For Hb Zürich M = 500 below, and 250 above, approximately half saturation with CO. The first may be a lower limit for the abnormal β subunits, because some of the normal α subunits would also be expected to take up CO below half saturation. The hemolysates of Hb Zürich, which contained about 15% abnormal β subunits, also showed an abnormally high M at low pCO, while for the hemolysates of Hb Sydney, which contained the same fraction of abnormal β subunits, all the points coincided within the error limits with those of Hb A. *Chironomus* Hb, which also lacks a distal histidine, has an intermediate value of M = 320.

DISCUSSION

The replacement of the distal histidine by arginine in Hb Zürich leaves a gap at the entrance to the heme pocket which allows sulfanilamides and other small reactive compounds easy access to the heme iron. Once there, its amide could act as a one-electron donor to the bound oxygen. If a second electron were

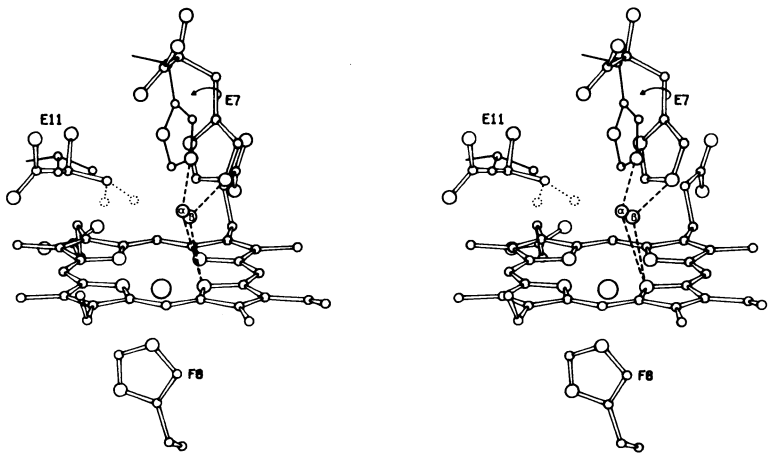

FIG. 4. Comparison of the β heme ligand pockets of deoxyHbs A and Sydney with the α heme ligand pocket of deoxyHb A. The orientation is similar to that of Fig. 3. The structure of the Hb Sydney β pocket is drawn with open perspective bonds; the replaced γ carbons of Val E11 are denoted in dotted outline. The structure of the α heme of deoxyHb A is superimposed on that of the β heme and the resulting positions of Val E11α and His E7α are shown as black stick bonds. The coordination of the water molecules of the Hb Sydney β pocket (β) and native α pocket (α) are shown with broken lines. The arrow represents the proposed rotation of the distal histidine of Hb Sydney.

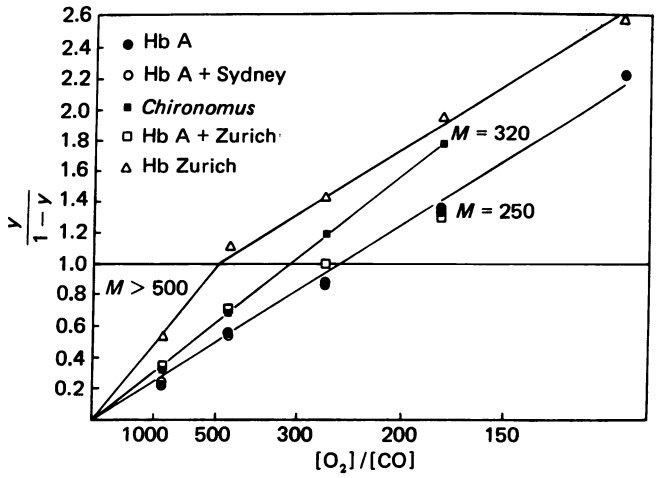

FIG. 5. Partition coefficients between CO and O_2. These were measured spectrophotometrically in a darkened tonometer of 270-ml capacity with a 1-cm pathlength optical cuvette sealed on to it. Initially the cuvette was filled with pure O_2 and 5 ml of a 150 μM (heme) solution of HbO_2 in 0.1 M K_2HPO_4 was added. Successive volumes of CO were injected with a gas-tight Hamilton syringe and the tonometer was rolled for 10 min at 20° after each injection. Difference spectra were recorded at 20° against a matched solution of HbO_2. The fraction of HbCO was determined from ΔA at 578 nm. y = [HbCO]/([HbCO] + [HbO_2]).

donated by the iron atom, the oxygen would be reduced to peroxide ion and the β heme would be oxidized to the met form. This reaction could initiate the denaturation of the Hb, formation of Heinz bodies, and hemolysis (23, 24). Wallace *et al.* (10) reported that, by contrast, the acid-catalyzed reductive displacement of superoxide by azide was slower in the β subunits of Hb Zürich than in Hb A, but we have since found this to have been an artefact due to contamination with CO. After careful removal of all CO the azide-promoted reaction is faster in Hb Zürich than in Hb A. The same is true of the rate of autoxidation of oxyHb Zürich *in vitro* (9). His E7 has been shown to have a pK of only 5.4, evidently due to its buried position (25). It has been suggested that at neutral pH its exchangeable proton is located on N_ϵ, forming a hydrogen bond with the heme-linked oxygen (25), but if this were true His E7 would promote, rather than inhibit, acid-catalyzed reductive displacement of superoxide at neutral pH. This inhibition shows that at neutral pH the exchangeable proton must be located on N_δ, facing the exterior of the molecule, and that acid pH accelerates oxidation by protonating N_ϵ.

The high oxygen affinity of Hb Zürich does not appear to be due to a low allosteric constant L, because its Bohr effect is normal (8) and the NMR spectrum of its deoxy form shows the exchangeable proton resonance at −9.4 ppm from HDO diagnostic of the quaternary deoxy (T) structure (G. Pifat and C. Ho, personal communication). Oxygen equilibrium curves show that the association constant of the first oxygen to combine with Hb Zürich is 7.5 times larger than that of Hb A, while that for the last oxygen is half that of Hb A (M. Ikeda-Saito, personal communication). This shows that in the T structure the oxygen affinity of the β subunits is abnormally high; it does not give their oxygen affinity in the R structure, because most of them would have combined with oxygen at an earlier stage of the reaction. In the R structure, therefore, only α chains will react with oxygen, and its abnormally low oxygen affinity must be due to the fact that the oxygen affinity of unconstrained normal α chains is lower than that of unconstrained normal β chains.

The second-order rate constant for the combination of CO with the T structure of Hb Zürich is much larger than that observed for the combination of CO with the R structure of Hb A (26). Why should the ligand affinity in the T structure be so high? The electron density map and the NMR spectrum of Hb Zürich suggest one mechanism that may contribute to the high affinity. Fig. 1 shows small negative peaks at the bottom of the β heme, which indicate that in Hb Zürich this heme may be turned counterclockwise relative to Hb A. Normally the bottom half of the heme is in contact with N_ϵ and C_ϵ of the distal histidine. The difference map suggests that these two atoms help to clamp the heme in its correct orientation so that their removal allows it to turn counterclockwise, i.e., to tilt further from the vertical. What would this tilt signify in terms of ligand affinity? Normally ligand binding and shortening of the Fe—N bonds is accompanied by a counterclockwise rotation, ligand dissociation and lengthening of the Fe—N bonds by a clockwise rotation (27, 28). The tilt in Hb Zürich would be in the direction normally associated with shortening of the Fe—N bonds. If this tilt were present also in deoxyHb Zürich it would relax the restraint on the globin that normally opposes the shortening of the Fe—N bonds associated with ligand binding and thus make the ligand affinity of the β hemes in the T structure abnormally high. The NMR spectrum of deoxyHb Zürich is consistent with this concept. DeoxyHb A shows a paramagnetically shifted proton resonance from the β hemes at −17.6 ppm from HDO, but in deoxyHb Kempsey and others that have the quaternary oxy (R) structure, this resonance lies at −15.3 ppm (29). In deoxyHb Zürich it lies at −15.4 ppm, i.e., in the same position as in the deoxyHbs in the R structure, even though it has the T structure (30). In HbCO Zürich the ring-current shifted resonance of one of the γ methyls of Val E11β is shifted further upfield than in HbCO A by 0.07 ppm (31), consistent with a closer approach of the methyl to the porphyrin brought about by the increased tilt. Taken together, these observations suggest that the β hemes in the T structure of Hb Zürich may be relaxed and therefore have as high a ligand affinity as they normally have in the R structure.

Maintenance of tension or restraint in the T structure by the distal histidine is an indirect effect and does not tell us what influence, if any, the distal histidine has on the iron ligand bonds in the R structure. The unchanged O—O stretching frequency in the β subunits of Hb Zürich indicates that the distal histidine can have but a small effect on the Fe—O_2 bond. On the other hand, all the evidence points to the conclusion that it weakens the Fe—CO bond. The partition coefficient between CO and O_2 of the β subunits of Hb Zürich is at least twice that of Hb A; the second-order rate constant of the reaction of β_{SH} Zürich chains with CO is larger than with β_{SH} A chains (26); and the CO stretching frequency of the β subunits of Hb Zürich is raised about a third of the way towards that of free CO iron porphyrins; their CO affinity is always higher than that of hemoglobin (32, 33). The distal histidine may affect CO binding by either electronic or steric effects. N_ϵ of the distal histidine is in contact with the CO carbon so that the sp^2 orbital of N_ϵ overlaps the empty π^* antibonding orbital of the carbon. $sp^2 \rightarrow \pi^*$ donation would weaken the CO bond and reduce the CO stretching frequency. It would also oppose $d\pi \rightarrow \pi^*$ donation from the iron to the CO, thus weakening the Fe—C bond, but this is a second-order effect. Sterically, the two distal residues conspire to push the CO off the heme axis (34). We cannot judge which of these two effects is the more important one in lowering the CO affinity.

We now come to Hb Sydney. Its abnormally high oxygen affinity at low partial pressures of O_2 and vice versa (13) can

now be explained by the water molecule bound to the distal histidine in deoxyHb Sydney, replacing the steric hindrance to ligand binding offered by Val E11 in deoxyHb A. Let us assume that in the absence of heme ligands this water molecule is present in both quaternary structures. In the *T* structure steric hindrance by the water replaces that normally offered by Val E11, which may be stronger, so that the oxygen affinity may be higher than that of Hb A. In the *R* structure of Hb A, Val E11 offers little steric hindrance, while that of the water molecule in Hb Sydney would remain as strong as in the *T* structure. Therefore, Hb Sydney in the *R* structure may have a lower oxygen affinity than Hb A. Though the presence of the water molecule compensates for the absence of Val E11, the price paid for this is readier autoxidation. Moreover, the gap left in the heme pocket must also be responsible for the instability of Hb Sydney that leads to hemolytic anemia. We now come to the binding of CO. The CO stretching frequency of the β subunits of Hb Sydney is shifted by one-sixth of the way towards that in free iron porphyrins, presumably due to the removal of some of the steric hindrance that normally tilts the CO off the heme axis. One would therefore have expected the Fe—CO bond to be strengthened, resulting in an increased partition coefficient between CO and O_2, but this has not been found. The association constant for $Hb_4(O_2)_3 + CO \rightarrow Hb_4(O_2)_3CO$ is also the same as for Hb A (J. A. Sirs, personal communication).

To summarize: in the β subunits the distal histidine helps to maintain the tension or restraint at the heme in the *T* structure and is therefore essential for the maintenance of the low oxygen affinity; by its low pK and the basic nature of its N_ϵ it opposes acid-catalyzed oxidation of the iron and it also protects the iron from other oxidizing agents; finally, it lowers the partition coefficient between O_2 and CO. Carriers of abnormal hemoglobins in which the distal histidine is replaced by another residue should be advised to refrain from smoking, and should not be given potentially oxidizing drugs. In the β subunits the distal valine lowers the oxygen affinity of the *T* structure by blocking the oxygen combining site at the iron atom; it also opposes autoxidation of deoxyHb by preventing the binding of a water molecule to N_ϵ of the distal histidine; it does not change the partition coefficient between oxygen and CO. Neither the histidine nor the valine appears to have a significant effect on the Fe—O_2 bond.

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