absorption spectra of superlattices.

The difference in the cathodic quantum yields between LMS electrodes with an undoped epilayer and a p-doped epilayer is due to photocurrent generated in the p-n junction that exists in the former configuration. The photocurrent from the configuration with the p-doped epilayer is derived only from the superlattice layers; a p-n junction does not exist here. The progressive loss of the peak structure in LMS electrode samples that have been progressively etched is, of course, additional proof that the pronounced cathodic photocurrent peaks arise only from the superlattice layers.

The higher quantum yields for the thin-barrier LMS compared to the thick-barrier LMS electrode result from enhanced tunnelling and miniband formation in the thin-barrier superlattice. The equilvalence of the photocurrent spectra for both SLS and LMS electrodes with either liquid or solid contacts shows that the behavior of superlattice electrodes described here is not related to the nature of the junction material.

Finally, in our previous publications on LMS and SLS electrodes,[1-3] we suggested that changes in the photocurrent action spectra with changes in the redox potential of electron acceptors in solution should serve as evidence for hot electron injection from the superlattice electrodes into solution. However, a detailed quantitative analysis of the kinetics of electron transfer from superlattice electrodes[7] together with the present results on SLS photoelectrodes indicates that this interpretation is not sufficiently general and may not be valid for our previous experimental conditions. Therefore, further experimental work is required to unequivocally establish the importance of hot electron transfer processes in superlattice electrodes.

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Direct Observation of Intermediate Ligation States of Hemoglobin

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Abstract: The ^{31}P NMR spectrum of partially liganded $HbPMe_3$ (HbAo hemoglobin) contains resonances at the normal chemical shift positions of the fully liganded species (R state), in addition to two resonances at intermediate positions. Analysis of the relative magnitude of these four peaks in the absence and in the presence of inositol hexaphosphate shows that PMe_3 binds preferentially to α chains and permits identification of the intermediate species. Plots of the fractional saturation of Hb versus the concentration of unbound PMe_3 exhibit markedly cooperative behavior as evidenced by the sigmoid nature of the binding curve and a large hill coefficient ($n = 2.3$). ^1H NMR studies of the high-field spectra of PMe_3 protons in $HbPMe_3$ are consistent with ^{31}P NMR results.

From the very beginning, the nature and role of intermediates in cooperative ligand binding to hemoglobin (Hb) has been difficult to define.[1] First there is the problem of direct observation of partially liganded states in unmodified hemoglobins. Low-temperature redox trapping of partially liganded HbCO has recently established their existence quantitatively and determined whether the site(s) of CO binding is in the α or β chains.[2] The more difficult problem of obtaining structural information about the intermediates and relating this to the mechanism of cooperativity has typically been approached with modified or hybrid hemoglobins.[3] These studies tend to be contradictory with respect to preferential ligand binding to the α or β chains,[4] but nevertheless, there is a growing evidence for preferred intermediate states. Many of these must have distinct tertiary structure and some[3d,5] may have quaternary structures that are distinct from the T or R state. In this paper, we show that ^{31}P NMR spectroscopy can be used as a direct probe of ligand binding to unmodified Hb. Two distinct intermediates are seen. Both appear to involve preferential binding to the α chains in the T state or in a modified quaternary structure T'.

Results and Discussion

The ligand used is a phosphine, PMe_3, which is small enough to complex Hb. We have previously shown that the iron-bound $^{31}PMe_3$ chemical shifts are sensitive to the presence of the globin: separated NMR signals can be observed for PMe_3 bound to the hemes of the α and β chains in the R state.[6] The ^{31}P NMR spectrum of partially liganded hemoglobin solutions is shown in

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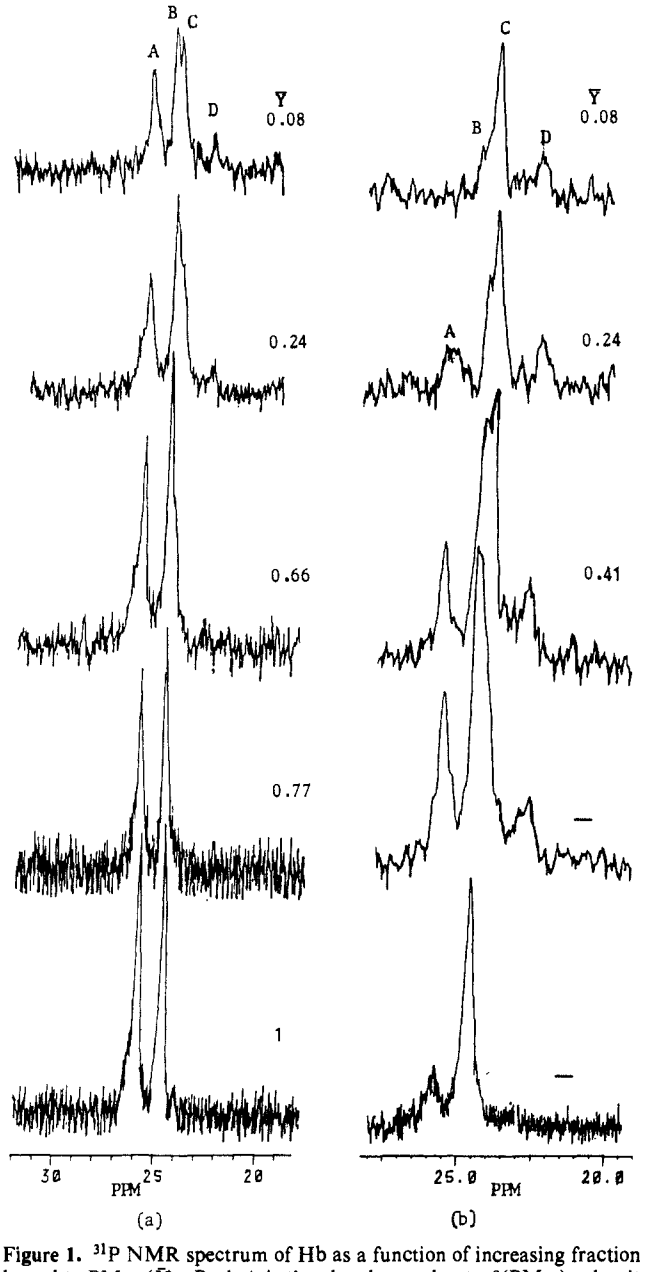

Figure 1. ^{31}P NMR spectrum of Hb as a function of increasing fraction bound to PMe_3 ($\bar{Y}$). Peak A is the absorbance due to $\beta(PMe_3)$ subunit (R state); B is due $\alpha(PMe_3)$ subunit (R state); C and D are due to the partially liganded intermediate species. (a) Without IHP; (b) with IHP (3.6 equiv/tetramer).

Figure 1a. In addition to the resonances characteristic of fully liganded $HbPMe_3$,[6] A (26 ppm, β chains) and B (24.8 ppm, α chains), two additional resonances C and D are present in the intermediate ligation range (8% → 24%). At low saturation ($\bar{Y}$ = 8%) the smaller of these resonances D appears 1.5 ppm upfield of the α subunit of $HbPMe_3$ resonance B (R state) whereas the larger intermediate resonance C appears only 0.3 ppm upfield of the resonance B. The positions of all four peaks are independent of the amount of trimethylphosphine addition, and both resonances C and D disappear at high saturation.

Reversibility of the PMe_3 ligation is accomplished by an equilibrium exchange experiment utilizing CO. For example, human hemoglobin saturated with PMe_3 exhibits absorbance measurements at λ_{max} = 434, 535, and 560 nm.[6a] Addition of CO leads to a decrease in the intensity of these peaks and the concomitant appearance of new peaks at 419, 540, and 569 nm

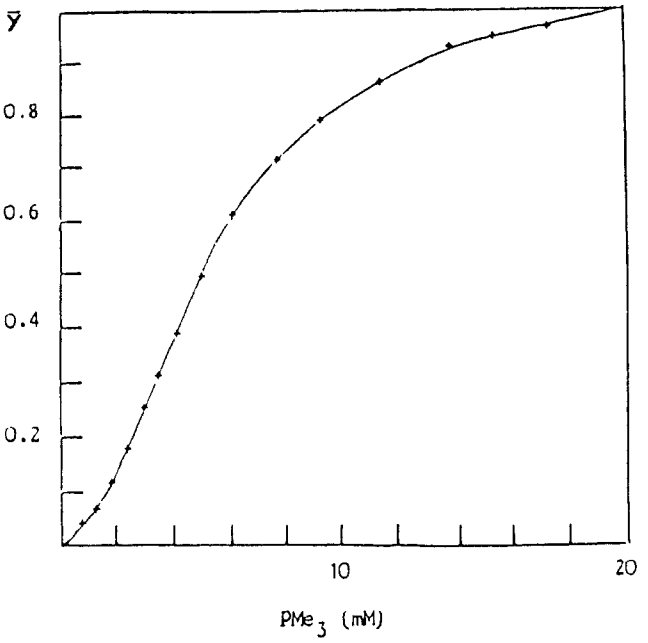

Figure 2. Observed equilibrium curve for the binding of PMe_3 to hemoglobin. The fractional saturations were determined from observations at two wavelenghts: λ_{max} = 535 and 561 nm (Hb = 1.5 mM) in 0.1 M Tris-HCl at pH 7.1, 20 °C.

which are known to correspond to CO bound Hb.[7] Determination of the absorption coefficient shows the absence of denaturation during this process.

Although phosphines have been used in previous studies on cytochrome P450[8] and chloroperoxydases,[9] the affinities of these ligands for hemoglobin have never been determined. The plot of the fractional saturation of hemoglobin versus the concentration of unbound ligand[10] is represented in Figure 2. As expected, the equilibrium data exhibit markedly cooperative behavior as evidenced by the sigmoid nature of the binding curve and a large Hill coefficient (n = 2.3).

Analysis of the ligand-binding data in molecular terms was dependent on the identification of the subunits influencing the NMR probe. Studies on the mercury-free α and β chains of Hb yield results that do not differ greatly from those of PMe_3 bound to these subunits in an intact tetramer. For the α(SH) subunit, a shift is observed 0.5 ppm upfield from resonance B (δ = 24.3 ppm) and for the β(SH) subunit (which exists as a tetramer) a shift is observed 0.5 ppm downfield from resonance A (δ = 26.5 ppm). The resonances resulting from $^{31}PMe_3$ bound to the α subunit of the different hemoglobins generally occur in the region of 24 ppm downfield from H_3PO_4; rabbit hemoglobin is an exception (22.9 ppm).[6] In contrast the resonance of $^{31}PMe_3$ bound to the β subunits of the various hemoglobins occurs in the region of 26 ppm downfield from H_3PO_4.[6] This leads us to suppose that the two high-field resonances (C and D) of the intermediates that are further upfield shifted from the α resonances in the R state may represent α-heme bound PMe_3. In order to ascertain which

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(10) PMe_3 (pK_a = 8.80) binds in its neutral form to Hb but is mainly under its protonated form in buffer solutions (pH 7.1). Titrations of the ferrous protein (1.5 mM), which were carried out in the presence of a slight excess of sodium dithionite under a nitrogen atmosphere, showed reproductible results; corrections due to the ligand basicity were not accomplished. A resonance at −2.8 ppm was observed by ^{31}P NMR and attributed to a $[PMe_3, H]^+$ complex: Lunsford, J. H.; Rothwell, W. P.; Shen, W. *J. Am. Chem. Soc.* **1985**, *107*, 1540–1547.

subunit is liganded in the intermediate species, the influence of the binding of inositol hexaphosphate (IHP) to human hemoglobin at intermediate stages of ligand saturation also has been studied by ^{31}P NMR (Figure 1b). In the presence of IHP, the chemical shift of PMe_3 bound to the α or β subunits of HbAo is unaffected but the intensity of each resonance is greatly modified. The presence of IHP at low saturation ($\bar{Y} = 8\%$) leads to an increase in the intensity of the intermediate resonances C and D, and the disappearance of resonance A (β subunits).[11] Organic phosphates,[12] such as 2,3-diphosphoglycerate (DPG) and inositol hexaphosphate (IHP), are known to bind between the β chains of deoxyhemoglobin and stabilize the T state. In 1970, using X-ray evidence, Perutz suggested that α chains have a higher affinity for ligand when hemoglobin is in the T state.[1c] The role of Val 67 in blocking the β-heme pocket has been confirmed by X-ray analysis of the deoxy structure.[13] Consequently, the intermediate resonances C and D must represent the PMe_3 bond to the α subunit(s) at intermediate stages of ligand saturation.[14] Thus, by stabilizing the T state of the hemoglobin tetramer, the presence of IHP would be expected to encourage preferential ligation of the α chains, a prediction that seems to be substantiated by this work on the binding of PMe_3. Moreover, the determination of the relative magnitude of NMR resonances A/B + C + D (β/α) without IHP suggests that in the early stage of ligand binding, the α chains of Hb exhibit higher affinity for PMe_3 than the β chains, even in the absence of IHP.

The comparison of the ^1H NMR spectra with ^{31}P NMR spectra of partially liganded $HbPMe_3$ confirms this observation. We previously asigned the prominent peak at −3.4 ppm to the α-PMe_3 subunit and the peak at −3.2 ppm to the β-PMe_3 subunit (Figure 3).[6b] There are two major conclusions that can be drawn from the ratio of the intensities of the α and β resonances in the ^1H NMR spectra. First, as expected the preference for the α chains is maintained. Second the ratio between the ligated α chains (calculated by using the sum of B (R state) and C + D (T or T′ state) resonances for α subunits) and the ligated β chains (resonance A) in the ^{31}P NMR spectra is in good agreement with the degree of the difference in affinity for PMe_3 between the α and β subunits observed in the ^1H NMR spectra. These conclusions are consistent with the extensive ^1H NMR studies on the binding of O_2 and CO to HbA reported by Ho et al., who have shown that in the presence of organic phosphates, the α chains have a much greater affinity than those of the β chains.[5a,15] While the ^{31}P NMR spectroscopy can be used as a direct probe of intermediate ligation states of hemoglobin, the ^1H chemical shift of ligated PMe_3 to α or β chain is independant of the Hb quaternary structure. This is quite unexpected since perturbations in the β heme pocket induced by a thiol reagent were detected in both ^1H and ^{31}P spectra.[6b] However, comparison of the $^{31}PMe_3$ chemical shift of $Fe(PMe_3)(TPP)(Im)$ and $Fe(PMe_3)(TPP)(2\text{-MeIm})$ reveals that the $^{31}PMe_3$ chemical shift of the latter ($\delta = 22.2$ ppm) is upfield shifted ($\Delta\delta = 1.5$ ppm) relative to the corresponding $^{31}PMe_3$ resonance of the unhindered imidazole complex ($\delta = 23.7$ ppm).[14] Similarities in chemical shift of model compounds and hemoglobin intermediates are coincidal but the relative values (the T state induces a highfield shift) are consistent with proximal histidine

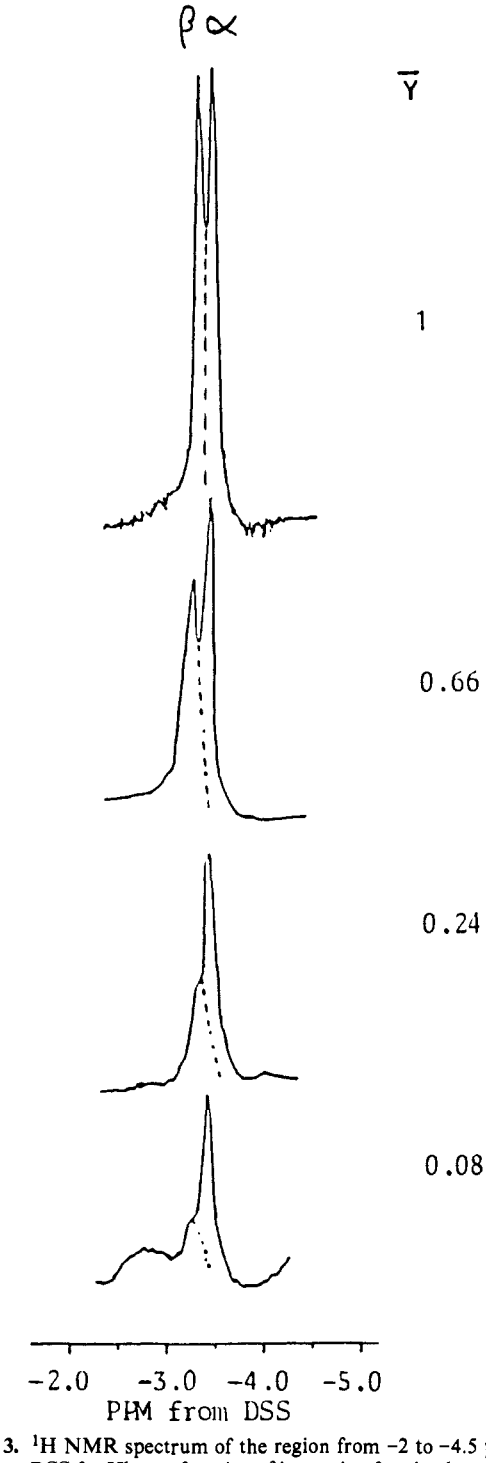

Figure 3. ^1H NMR spectrum of the region from −2 to −4.5 ppm highfield from DSS for Hb as a function of increasing fraction bound to PMe_3 ($\bar{Y}$). Dotted lines illustrate the methods for determining the area ratios of the two resonances.

constraint in the mechanism of cooperativity.[16]

The strong, preferential binding to the α chain in the T state with respect to the β chain was recently supported by crystallographic studies of doubly oxygenated crystals which show a T state hemoglobin with oxygen bound to the α chains but not to the β chains.[5c] The Hill coefficient for the oxygenation of HbA is about 3 and that for the binding of PMe_3 is only 2.3. Thus, one would expect to accumulate more ligation intermediates in the binding of PMe_3 to HbA than in the binding of O_2 to HbA.[17] The present

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results support also the prediction of the allosteric model, recently proposed by Gill et al., that carbon monoxide ligation occurs first at α chains in the T state and then at the β chains after the conformational transition to the R state.[18] Like CO binding which is also cooperative, PMe_3 binding is turning out to be a useful model for the physiologically important binding of O_2.

Experimental Section

Adult human hemoglobin was prepared in the usual manner from fresh whole blood samples obtained from the local blood bank.[6] The α and β subunits of human hemoglobin were separated and purified as described by Geraci et al.[19] A stock solution of sodium inositol hexaphosphate (IHP) (obtained from Sigma as the sodium salt) was added to the hemoglobin samples to a final concentration of 3.6 molar excess per Hb tetramer when required. Deoxygenated human hemoglobin solutions (1.5 mM) in the Tris-HCl buffer (0.1 M, pH 7.1) were introduced in argon-filled NMR tubes. A small amount of sodium dithionite ($\approx$1 mg) was injected in the deoxy sample to be sure it was completely deoxygenated. Small aliquots (10-60 μL) of standard phosphine solution were then added. Stock solutions containing 0.26 M PMe_3 were prepared by dissolving 40 mg of fresh PMe_3 in 2 mL of degassed water.

Visible and NMR spectra were observed concurrently at various stages of ligand binding. NMR samples were transferred anaerobically to a flushed cell usuable for optical absorbance measurements on samples with high absorbance. The percentage of ligation $\bar{Y}$ was determined by measurement at λ_{max} = 535 (ϵ 22.8 mM^{-1} cm^{-1}) and 560 nm (ϵ = 24.6 mM^{-1} cm^{-1}) with extinction coefficients taken from the fully liganded form of the protein. The ^1H and proton decoupled ^{31}P NMR spectra were recorded in a pulse Fourier transform mode with a Brucker AM 300 WB spectrometer operating at 300 MHz for ^1H and at 121.49 MHz for ^{31}P. In ^1H NMR experiments, lines were drawn along the sides of each resonance, giving an essentially triangular representation of the area of each resonance. The area was then measured with a planimeter. The probe temperature was maintained at 20 °C. ^1H and ^{31}P chemical shifts were respectively measured with respect to (trimethylsilyl)propanesulfonic acid and H_3PO_4 external standards. The chemical shift scales are defined as positive in the low-field direction with respect to the references.

Ultraviolet-visible spectra were recorded with a Jobin Hitachi spectrometer. The pH values of the samples were measured on a Bioblock Model 93301 pH meter equipped with a Schott electrode (A 90438).

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NMR Studies of Nucleic Acids. Deuterium Isotope Effects on ^{13}C Chemical Shifts in Hydrogen-Bonded Complexes of Pyrimidines and Purines

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Abstract: ^{13}C NMR spectra were recorded for chloroform solutions of 2',3'-*O*-isopropylidene-5'-*O*-acetyladenosine (**1**), 2',3',5'-*O*-tribenzoyluridine (**2**), 2',3'-*O*-isopropylidene-5'-*O*-acetyluridine (**2a**), 2',3'-*O*-isopropylidene-5'-*O*-(*tert*-butyldimethylsilyl)guanosine (**3**), and 2',3'-*O*-isopropylidene-5'-*O*-(*tert*-butyldimethylsilyl)cytidine (**4**) in which the imino hydrogens were partially exchanged with deuterium. Upfield two-bond deuterium isotope effects (DIE) on ^{13}C chemical shifts were detected under conditions of slow exchange as multiple peaks for the appropriate resonances and ranged in magnitude from 40 ppb for the amino interaction with C2 in guanosine to 217 ppb for the imino interaction with C4 in the uridine self-association dimer. ^{13}C chemical shifts and DIEs for **2** were measured at 12 different concentrations from 219 to 231 K. The data were used in an iterative procedure to estimate chemical shifts at C2 and C4 for monomeric and dimeric forms of **2**, equilibrium constants and enthalpies for self-association, and the distribution of isomeric self-association dimers. Enthalpies for formation of hydrogen bonds to C2 and C4 in **2** were similar, ΔH = -1.8 kcal/mol. DIEs at C2 and C4 increased upon formation of a hydrogen bond to the carbonyl oxygens. The maximal increase for each center was estimated to be 90 ppb. Small increases were also observed in DIEs when nucleosides **1-4** were mixed with their complementary bases.

Hydrogen bonds are responsible for much of the specificity seen in the conformations of proteins[1] and nucleic acids,[2] in enzyme-substrate binding,[3,4] and in replication,[5] transcription,[6] and translation[7] of the genetic code. Of the many different techniques that have been employed to study hydrogen bonds during the past 20 years, NMR spectroscopy has proved to be especially useful. The chemical shifts of magnetically active nuclei, such as ^1H, ^{13}C, and ^{15}N, often show substantial displacements when groups bearing these atoms engage in hydrogen bonding.[8-10] This information is useful for detecting specific hydrogen bonds and studying the thermodynamic properties of hydrogen-bonded complexes.

NMR techniques have been widely used to study the structures of nucleic acids. In a pioneering study, Katz and Penman[11]

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