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Site and Mechanism of Covalent Binding of 4-Dimethylaminophenol to Human Hemoglobin, and Its Implications to the Functional Properties

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SUMMARY

4-Dimethylaminophenol, after i.v. injection, rapidly forms ferrihemoglobin and has been successfully used in the treatment of cyanide poisoning. The catalytic ferrihemoglobin formation is terminated by thioether formation of oxidized 4-dimethylaminophenol with reduced glutathione or cysteine 93β of hemoglobin. Hereby the physiological functions of human hemoglobin are markedly altered. After binding of two molecules of 4-dimethylaminophenol to tetrameric hemoglobin, the rate of autoxidation is increased about 6-fold. The oxygen affinity is 10 times higher than normal, the Hill coefficient is diminished nearly to unity, and the Bohr effect is reduced by about 50%. The physiologically important allosteric regulation of the oxygen affinity by 2,3-diphosphoglycerate is abolished, and the binding of 2,3-diphosphoglycerate to deoxyhemoglobin no longer functions. By molecular sieving, two alkylated hemoglobins were separated: a hemoglobin fraction with an unchanged low tetramer dimer dissociation, normal electronic spectra, and normal digestibility by carboxypeptidase A; and a second fraction with a high degree of dissociation, altered electronic spectra, and impaired digestibility. A tryptic peptide was isolated containing cysteine 93β and histidine 146β cross-linked by an arylic compound missing the dimethylamine label. The following reaction mechanism is concluded: Oxyhemoglobin catalyzes the oxidation of 4-dimethylaminophenol, and the oxidation product, presumably *N,N*-dimethylquinonimine, is bound covalently to cysteine 93β by a thioether linkage. This adduct is unstable and autoxidizes further with the liberation of dimethylamine. The resulting quinoid thioether electrophilically attacks the COOH-terminal histidine of the β-chain, thereby forming an intramolecular cross-link. By this latter reaction, hemoglobin lacks allosteric transition upon ligation and is obviously frozen in its quaternary *R*-state.

INTRODUCTION

DMAP[1] has proven its superiority as an antidote in cyanide poisoning in dogs and man (1, 2) and is marketed as a drug in the Federal Republic of Germany. DMAP is most effective after i.v. injection and forms substantial amounts of ferrihemoglobin in less than 1 min, thereby trapping cyanide within red cells (3). Previous studies with purified human hemoglobin have shown that DMAP catalytically transfers electrons from ferrohemoglobin to oxygen (4). This highly active catalytic electron transfer would lead to life-threatening methemoglobinemia if the catalytic process did not cease in good time. There are three basic reaction pathways by which DMAP is readily eliminated and ferrihemoglobin formation terminated: (a) conjugation of DMAP in the liver (5–7); (b) thioether formation of DMAP with GSH (8); and (c) covalent binding of DMAP to hemoglobin (9).

Covalent binding of reactive intermediates of foreign compounds to cellular macromolecules has generally been associated with toxicity or even carcinogenicity. Often, the consequences of such a covalent binding have been described only phenomenologically, and the basic molecular mechanism underlying the observed toxicity remained obscure. When we studied the alterations in the physiological functions of hemoglobin to which DMAP had bound covalently, we were able to correlate the observed alterations with a molecular structure in which the alkylated hemoglobin is "frozen" in its quaternary R-state. From investigations which have been previously communicated elsewhere, we reasoned that DMAP is almost exclusively bound to the reactive cysteine residues in position 93 of human β-chains. However, the dramatically altered properties of this alkylated hemoglobin cast some doubt on a simple thioether forma-

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[1] The abbreviations used are: DMAP, 4-dimethylaminophenol; pCMB, *p*-chloromercuribenzoic acid; CPA, carboxypeptidase A; DPG, 2,3-diphosphoglycerate; IHP, inositol-hexaphosphate; Hb, hemoglobin; COHb, carbon monoxide hemoglobin; $HbFe^{3+}$, ferrihemoglobin; Hb_{DMAP}, hemoglobin with covalently bound DMAP; Hb_{PMB}, hemoglobin after reaction with pCMB.

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tion of the oxidized DMAP with cysteine 93β (9). We therefore reexamined in more detail the functional properties of human hemoglobin to which DMAP was bound covalently, and investigated the site and mechanism of these reactions.

MATERIALS AND METHODS

DMAP. DMAP, [$^{14}CH_3$]DMAP (specific activity 1.2 mCi/mmole), and [U-^{14}C-*phenyl*]DMAP (specific activity 5 mCi/mmole were prepared by Farbwerke Hoechst; the radiochemical purity was >98%.

Hemoglobin. Hemoglobin was prepared from freshly drawn venous blood which was always obtained from the same nonsmoker (COHb). The red cells were washed three times with 5 volumes of phosphate buffer (pH 7.4). The packed cells were lysed in 2 volumes of distilled water by ultrasonic treatment, and hemoglobin was purified by gel and anion exchange chromatography as described elsewhere (10). This hemoglobin preparation consisted essentially of "stripped" HbA with less than 1% phosphate per tetramer.

Hb_{DMAP}. Hb_{DMAP} was prepared by incubation of purified hemoglobin (3 mM Fe) with 1.5 mM DMAP at pH 7.4, 37° for 30 min under O_2. Under these conditions, virtually all DMAP was covalently bound to hemoglobin. The solution was then exhaustively dialyzed against the desired buffer.

Hb_{PMB} was prepared by incubation of purified hemoglobin with pCMB (moles per mole of Fe) under the same conditions as described for Hb_{DMAP}. Complete blockage was checked by the method of Boyer (11).

$HbFe^{3+}$. $HbFe^{3+}$ was prepared by addition of a 1.5 M excess of potassium ferricyanide to purified hemoglobin at pH 7.4. After a 30-min reaction, the mixture was chromatographed on Sephadex G-25 with 0.1 M Tris-HCl (pH 7.4).

Total hemoglobin and ferrihemoglobin. Total hemoglobin and ferrihemoglobin were measured by the method reported by Kiese (12) or as the carbon monoxide derivative in the presence of sodium dithionite, using a molar extinction coefficient of 13.4×10^3 at 569 nm and 191×10^3 at 419 nm, respectively (13).

Spectroscopic determinations. Spectroscopic determinations were carried out on a Zeiss PMQ 3 spectrophotometer. The accurate reproducibility of the wavelengths was controlled by measuring the absorption of a blue glass standard with a high $\Delta E/\Delta$nm ratio between 580 and 540 nm. Electronic spectra were recorded with a Cary 118 spectrophotometer.

Deoxygenation and ferrihemoglobin reduction. Deoxygenation and ferrihemoglobin reduction of the hemoglobin preparations was achieved by repetitive evacuation and flushing with nitrogen of the hemoglobin solution and subsequent addition of sufficient sodium dithionite under nitrogen. The solution was then passed through a column of Sephadex G-25 (2.5 cm inner diameter × 25 cm) equilibrated with the desired buffer. The whole procedure, including the filling of cuvettes or tonometers, was performed under nitrogen. If reoxygenation of the deoxygenated hemoglobin solutions was necessary, the eluate of the column was cooled on ice and then flushed with pure oxygen. Complete deoxygenation and oxygenation were checked spectroscopically. The ferrihemoglobin content in the deoxygenated samples was always less than 1%.

Oxygen equilibrium curves. Oxygen equilibrium curves were obtained essentially by the spectroscopic method of Rossi-Fanelli and Antonini (14). After introduction of 3 ml of deoxygenated ferrihemoglobin (60 μM Fe) in a tonometer (65 ml, filled with nitrogen, equipped with a cuvette, light path 1 cm), absorbancies at 577 nm and 548 nm were determined. Complete deoxygenation was indicated by a ratio of absorbancies 577 nm/548 nm = 0.75, and complete oxygenation by a ratio 577 nm/548 nm = 1.22. A defined volume of air was then introduced with a syringe and, after equilibration at 25°, spectroscopic readings were taken. Finally, the ferrihemoglobin content was determined, being always less than 7%. Partial oxygen pressures were calculated according to the method of Rossi-Fanelli and Antonini (14), and the degree of oxygenation from the ratio of absorbancies. Each point of the oxygen equilibrium curves was obtained from at least duplicate measurements with separate samples, which agreed within 2%. Only samples with less than 5% of ferrihemoglobin after reoxygenation were used for calculation.

Tryptic digestion. For tryptic digestion, the β-globin was denatured in 8 M urea (pH 5 at 60°) for 45 min according to the method of Liebold and Braunitzer (15). After dialysis against distilled water, the solution was flushed with nitrogen, adjusted to pH 9 with sodium hydroxide, and kept constant at pH 9 by means of a pH-stat (Metrohm, Filderstadt) during digestion with 5 mg of trypsin at 37°. After a 3-hr digestion, sodium hydroxide consumption ceased and was not stimulated by further addition of trypsin.

Digestion with carboxypeptidase A (EC 3.4.12.2). The hemoglobin preparation (2.5 ml, 0.3 μmole in 0.2 M sodium bicarbonate pH 9.2) was incubated with 0.1 mg of carboxypeptidase A at 37° under carbon monoxide, to avoid autoxidation. At different time intervals, samples were taken and precipitated with perchloric acid; the supernatants were analyzed for free amino acids.

Amino acid analysis. Amino acid analysis was performed with a Multichrom M amino acid analyzer (Beckman, München), using the lithium picobuffer system of Pierce, Eurochemie (Rotterdam). Acid hydrolysis of peptides was performed in 6 N hydrochloric acid at 110° for 24 hr under nitrogen.

Covalent binding. Covalent binding of radioactive compounds to hemoglobin was determined in precipitates after several washings with 0.6 M trichloroacetic acid and methanol. Precipitates were dissolved in Soluene 100 (Packard), and the radioactivity was measured as described below. Essentially the same results were obtained from measurements of the difference between the radioactivity in 0.6 M tricholoracetic acid supernatants and the radioactivity of the whole sample.

Radioactivity. Radioactivity in colorless solutions was measured in Bray's solution with a Packard Tri-Carb scintillation spectrometer using the external standard. Radioactivity in hemoglobin-containing solutions was determined in Insta-Gel (Packard) after decolorization in Soluene 100 and H_2O_2. All results were corrected for recovery and background radiation.

Assays. DMAP in ether extracts after thin-layer chromatography on silica gel plates with chloroform/methanol (95:5) was determined by reading the UV absorbance at 247 nm (log E = 4.07). [^{14}C]Dimethylamine and [^{14}C]formaldehyde were determined as reported elsewhere (4). Organic phosphates were determined according to the method of Ames and Dubin (16). Chemicals were obtained from commercial sources and were of the purest grade available.

RESULTS

Reaction of DMAP with the reactive SH groups of hemoglobin. When purified human hemoglobin (1 mM Fe) was incubated with surplus DMAP (U-^{14}C-*phenyl*) at pH 7.4 and 37°, covalent binding of DMAP proceeded biphasically. As calculated on an iron basis, up to one-half of the stoichiometric amount of DMAP was completely bound within 5 min, whereas additional DMAP was bound only slowly. When the reactive SH groups of human hemoglobin were blocked with pCMB prior to reaction with DMAP, covalent binding was greatly reduced. Moreover, titration of the SH groups in human hemoglobin after reaction with various amounts of DMAP revealed a stoichiometric decrease in the titratible SH groups. All of these data pointed to the accessible SH groups (13) of the cysteine residues in position 93 of the β-chains as the favored binding site for DMAP.

Autoxidation of Hb_{DMAP}. Purified human hemoglobin (3 mM) which had bound 1.5 mM DMAP was reduced by

sodium dithionite under nitrogen, and the ferrohemoglobin was chromatographed on Sephadex G-25 with 0.2 M phosphate under nitrogen and reoxygenated with pure oxygen on ice. Untreated hemoglobin and hemoglobin which had reacted with pCBM were processed identically.

The apparent initial rates of autoxidation of untreated hemoglobin, Hb_{PMB}, and Hb_{DMAP} were 0.91, 1.27, and 6.2 μM $HbFe^{3+}$/min, respectively. At the lower oxygen pressure of 22 mm Hg, the corresponding rates were 1.7, 2.0, and 8.3 μM $HbFe^{3+}$/min. Hemoglobin pretreated with *N*-ethylmaleimide autoxidized with rates similar to those for Hb_{PMB}. Thus, the increased rate of autoxidation of the DMAP-modified hemoglobin was not explained solely by SH group blockage.

Oxygen affinity. Oxygen equilibrium curves were determined in 0.2 M sodium phosphate, pH 7.4 at 25°. All hemoglobin preparations were carried through the same deoxygenation procedure. The oxygen affinity of Hb_{DMAP} was increased about 10-fold ($pO_2$50: Hb_{DMAP} = 0.9 mm Hg; Hb_{native} = 8 mm Hg). The Hill coefficient was decreased from 2.8 to 1.2, indicating a lack of homotropic effects in cooperativity. In contrast, Hb_{PMB} showed only a slightly enhanced oxygen affinity ($pO_2$50 = 6.5 mm Hg) and moderately reduced cooperativity (1.7).

Alkaline Bohr effect. The oxygen affinity of hemoglobin after reaction with DMAP was determined in 0.2 M sodium phosphate in the range between pH 6 and 9 at 25°. Half-saturation was determined graphically from Hill plots which had been obtained from duplicate measurements at six different oxygen pressures ranging from about 20% to 80% saturation. Figure 1 (*upper panel*) shows the plot of log $pO_2$50 versus pH for native hemoglobin and Hb_{DMAP}. The curves in the *lower panel* of Fig. 1 were calculated by using Wyman's equation for linked functions simplified to $\Delta \log p_{50}/\Delta pH = \Delta H^+$ (13). The *symbols* in the *lower panel* represent data from direct back-titration measurements with sodium hydroxide after reaction of deoxyhemoglobin with carbon monoxide in 0.1 M sodium chloride at 25°. Carbon monoxide was favored instead of oxygen to avoid autoxidation of Hb_{DMAP}. With both independent methods, a lowering of the alkaline Bohr effect by nearly 50% was observed for Hb_{DMAP}.

Effects of organic phosphates on the oxygen affinity. The marked increase in oxygen affinity with loss of cooperativity in the case of Hb_{DMAP} pointed to a severe disturbance in the quaternary structure of hemoglobin. We therefore examined the ability of organic phosphates, DPG and IHP, to shift the equilibrium from the apparent *R*-state to the *T*-state in Hb_{DMAP}.

The oxygen equilibrium curves of hemoglobin in 0.1 M Tris-HCl, pH 7.4 at 25°, in the presence and absence of organic phosphates are shown in Fig. 2. Whereas DPG had no effect on the oxygen equilibrium curve of Hb_{DMAP}, IHP shifted the oxygen equilibrium curve to the right with a $\Delta \log pO_2$50 of only 0.37, in contrast to 1.1 in the case of native hemoglobin. The cooperativity of Hb_{DMAP} was slightly increased by IHP, as indicated by an increase in the Hill coefficient from 1.3 to 1.45. In contrast, IHP lowered the Hill coefficient from 2.75 to 2.4 in native hemoglobin.

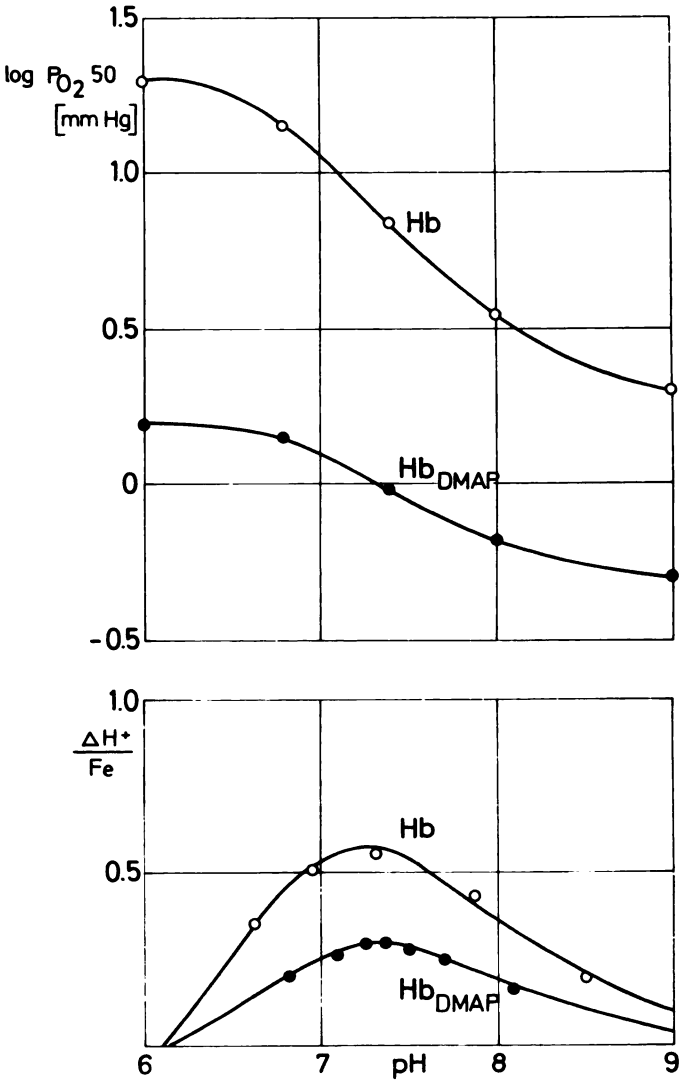

FIG. 1. *Oxygen Bohr effect*

Upper panel. Log $pO_2$50 versus pH with stripped native human hemoglobin and hemoglobin after reaction with DMAP in 0.2 M phosphate, pH 6–9 at 25°.

Lower panel. ΔH^+ versus pH: smooth curves calculated from $\Delta \log pO_2$50/$\Delta pH = \Delta H^+$. Symbols indicate data from direct back-titration measurements after reaction of deoxyhemoglobin with carbon monoxide in 0.1 M NaCl at 25°C.

Binding of organic phosphates to hemoglobin. Since DPG had no effect on the oxygen affinity of Hb_{DMAP}, it was of interest to examine whether Hb_{DMAP} was able to bind DPG in the absence of ligands and at a pH below neutrality (17). Hemoglobin solutions (2 ml, containing 1.5 mM Fe, 0.75 mM DPG or IHP, in 0.1 M ammonium acetate, pH 6) were deoxygenated and, after addition of a few crystals of sodium dithionite, chromatographed on a column of Sephadex G-50 fine (2.5 cm inner diameter × 25 cm) with ammonium acetate (pH 6) under nitrogen. Fractions (2.5 ml) were collected and assayed for hemoglobin and phosphate. Figure 3 shows the elution profile of hemoglobin and DPG or IHP, respectively. Whereas IHP was significantly bound to Hb_{DMAP}, binding of DPG to Hb_{DMAP} was nearly abolished.

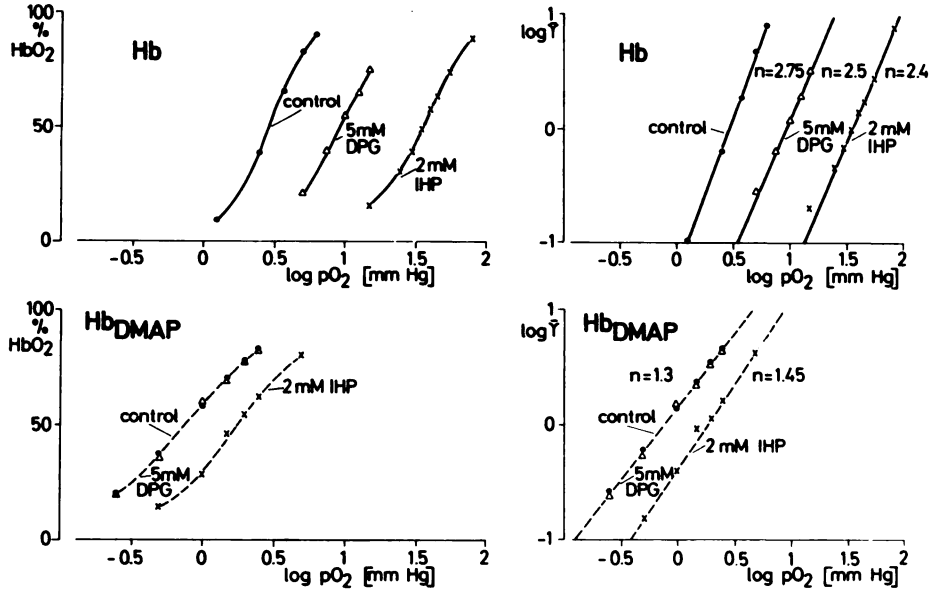

FIG. 2. *Effects of organic phosphates on oxygen equilibrium curves and Hill plots*

Determinations with stripped human hemoglobin, 1 mg/ml, in 0.1 M Tris-HCl, pH 7.4 at 25°. Upper panel, untreated hemoglobin; lower panel, hemoglobin after reaction with DMAP. ●, In the absence of organic phosphates; △, in the presence of 5 mM DPG; ×, in the presence of 2 mM IHP.

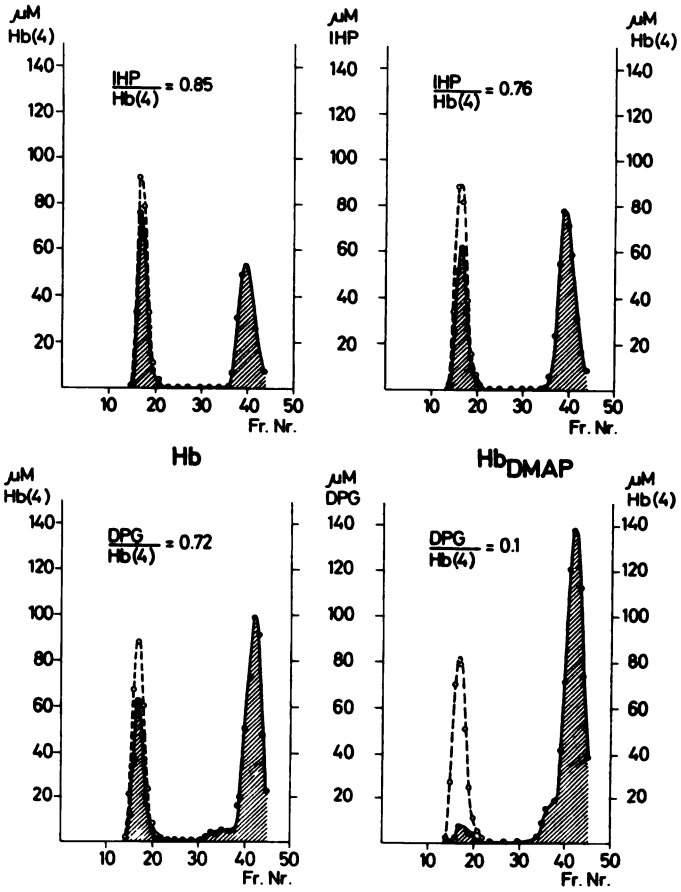

FIG. 3. *Binding of organic phosphates to hemoglobin*

Elution profiles of column chromatographies on Sephadex G-50 of incubates of stripped deoxyhemoglobin with the 2-fold stoichiometric amounts of DPG or IHP, respectively, in 0.1 M ammonium acetate (pH 6) under nitrogen. The ratios of bound phosphate to tetrameric hemoglobin are inserted.

Electronic spectra. The most prominent differences between electronic spectra of Hb_{DMAP} and native hemoglobin were observed with ferrihemoglobin in the region of the α- and β-band in addition to the obvious differences in the UV region. The difference spectrum between $HbFe^{3+}$ and $HbFe^{3+}_{DMAP}$ showed elevated α- and β-bands which were shifted by 15 nm to a lower wavelength. The influence of IHP on the spectrum of $HbFe^{3+}_{DMAP}$ in 0.1 M Tris-HCl (pH 7.4) was very small as compared with native ferrihemoglobin (Fig. 4). The effect of IHP on deoxy Hb_{DMAP} is shown in Fig. 5. In the Soret region, the maximal absorbance at 430 nm was elevated by about 3% in the presence of IHP, whereas IHP had no such effects on the electronic spectrum of native deoxyhemoglobin.

Isolation of tryptic peptides bearing the DMAP label. These results indicated the absence of changes in the quaternary structure of Hb_{DMAP} upon ligation, which casts some doubt on a simple thioether formation of the oxidized DMAP with cysteine 93β. Therefore, we reexamined the binding site using a radioactive DMAP preparation. Human hemoglobin (3 mM Fe) was incubated with 1.5 mM DMAP (U-^{14}C-*phenyl*; 0.81 μCi/μmole) under oxygen at 37° for 30 min. The mixture was then flushed with carbon monoxide for 10 min, and the ferrihemoglobin was reduced with a small excess of sodium dithionite followed by chromatography on Sephadex G-25 with 80 mM sodium chloride under carbon monoxide. The α- and β-chains were separated on CM_{52}-cellulose after reaction with pCMB according to the method of Bucci and Fronticelli (18). The α-chains contained virtually no radioactivity, whereas the β-chains exhibited 2-fold specific activity as compared with the tetrameric hemoglobin. To split off the heme, the pooled β-chain-containing fractions were dialyzed against distilled water and treated with acidic methylethyl ketone according to the method of Teale (19). After dialysis against distilled water, the β-globin was lyophilized. From 15 g of hemo-

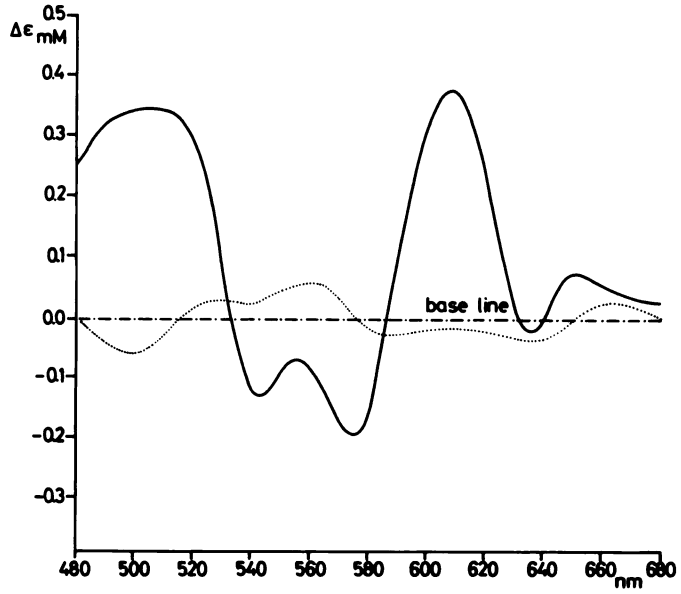

FIG. 4. *Influence of IHP on the electronic spectra of $HbFe^{3+}$ and $HbFe^{3+}_{DMAP}$*

Difference spectra in 0.1 M Tris-HCl (pH 7.4) after addition of a 50-fold stoichiometric amount of IHP. ——, $HbFe^{3+}$; ····, $HbFe^{3+}_{DMAP}$.

globin, 2 g of β-globin were obtained with a specific activity of 50 μCi/g of protein, corresponding to 0.99 mole of DMAP per mole of β-globin. Tryptic digestion of this β-globin consumed only 11 Eq of sodium hydroxide instead of the theoretical 14, indicating incomplete digestion. Chromatography of the peptides on conventional ion exchange resins was unsuccessful because adsorption to the polystyrene matrix resulted in deleterious spreading of radioactive material. Separation of the peptides succeeded only on hydrophilic matrices. Chromatography on Sephadex LH-20 (2.5 cm inner diameter × 250 cm) with 1 M acetic acid separated high- and low-molecular radioactive peptides.

The high-molecular fraction was further chromatographed on Sephadex G-50 fine (1.4 cm inner diameter × 250 cm) with 1 M acetic acid followed by chromatography on SP-Sephadex C-25 (0.8 cm inner diameter × 60 cm) equilibrated with 50 mM sodium acetate (pH 5). About 75% of the applied radioactivity was eluted in a single peak with a linear salt gradient from 0 to 5 M sodium chloride in 50 mM sodium acetate (pH 5). This fraction, Peptide I, was finally desalted on Sephadex G-25 and lyophilized (yield 47%). The low-molecular fraction was chromatographed on SP-Sephadex C-25 (0.8 cm inner diameter × 60 cm) equilibrated with 50 mM sodium formate (pH 3.25). During elution with a gradient from the equilibration buffer to 500 mM sodium acetate (pH 5.5), two major radioactive peptides were separated and desalted, Peptide II (4% yield) and Peptide III (2% yield).

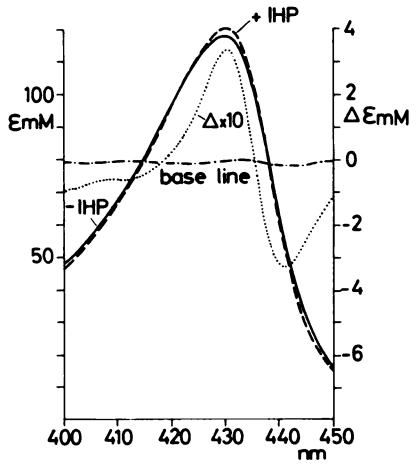

FIG. 5. *Influence of IHP on the electronic spectrum of deoxyHb_{DMAP}*
For conditions see Fig. 7.

Two-dimensional chromatography, "fingerprinting" according to Ingram (20) and Baglioni (21), indicated that the three peptides were chemically and radiochemically pure. After dansylation, hydrolysis, and chromatography (22), the NH_2 terminus of Peptide II was revealed to be glycine. The same was found with Peptide III, whereas Peptide I exhibited two dansylated amino acids in equal amounts, glycine and valine.

Amino-acid analysis of the completely hydrolyzed peptides indicated that only Peptide II was the expected tryptic peptide containing the residues 83–95β, and Peptide III was the incompletely digested "core" peptide (15), containing the residues 83–104β. In contrast, Peptide I exhibited an unexpected amino acid composition which fit only to a β-chain fragment containing residues 83–104β and 133–146β with one histidine missing, however. None of the three peptides liberated cysteine (detectable as cystine) after hydrolysis. Oxidation with performic acid according to the method of Hirs (23) failed to produce cysteic acid. Hence, thioether formation of DMAP with cysteine 93β was assumed.

Treatment of the three radioactive peptides with Raney nickel (8) did not liberate ether-extractable radioactive compounds from Peptide I, whereas Peptide II released 86% and peptide III 76% of a single radioactive compound which proved to be DMAP (thin-layer chromatography, UV). These results indicated that Peptide II and Peptide III, containing cysteine 93β, had bound DMAP via a thioether linkage. Peptide I, on the other hand, was presumably cross-linked by DMAP between cysteine 93 and the COOH-terminal region of the β-chain. This bond was not cleaved by Raney nickel. From reactions of DMAP with glutathione (8), we concluded that DMAP, after thioether formation with cysteine 93β, had been further oxidized to yield a quinonimine which reacted with amino groups in a 1,4-addition mode (4). Since the *N,N*-dimethylquinonimine of DMAP is easily hydrolyzed to benzoquinone and dimethylamine (4), dimethylamine liberation should indicate such a transient quinonimine structure.

Secondary changes in hemoglobin after covalent binding of DMAP. When either [$^{14}CH_3$]DMAP or [^{14}C-*phenyl*]DMAP (1.5 mM each) was incubated with hemoglobin (3 mM) in 0.2 M sodium phosphate, pH 7.4 at 37°, under various oxygen pressures, radioactive compounds in the supernatant (after precipitation with trichloroacetic acid) rapidly decreased. As shown in Fig. 6, the ring label of DMAP was quickly and irreversibly bound to hemoglobin, irrespective of the oxygen pressure. In contrast, binding of the methyl label was only some 70% and decreased slowly during further incubation. The velocity of liberation of acid-soluble compounds bearing the methyl label clearly depended on oxygen pressure.

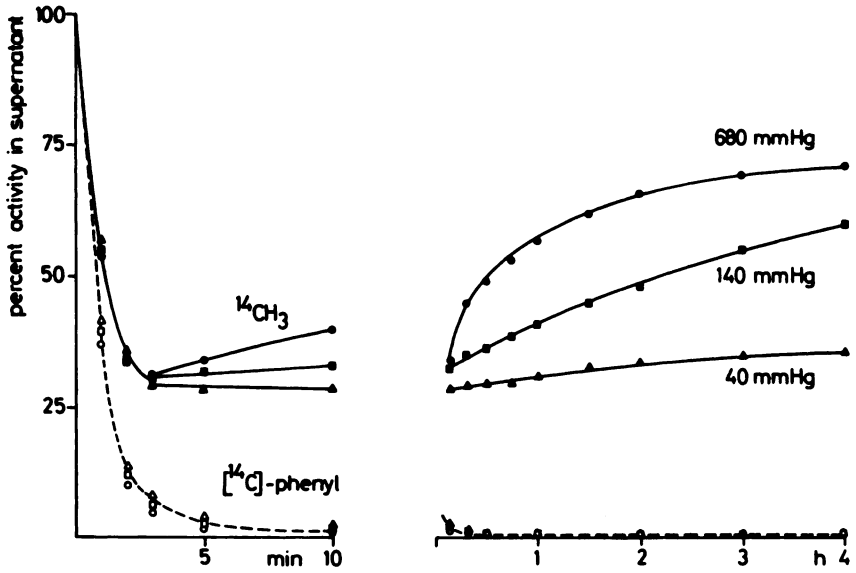

FIG. 6. *Influence of oxygen pressure on the release of $^{14}CH_3$-labeled metabolites of DMAP after binding to hemoglobin*

Purified human hemoglobin (3 mM) was incubated with 1.5 mM DMAP (labeled either in the ring or in the methyl groups) in 0.2 M sodium phosphate, pH 7.4 at 37°, under various oxygen pressures. After precipitation of the hemoglobin with trichloroacetic acid, the radioactivity was measured in the supernatant.

More than three-fourths of the acid-soluble radioactive compounds consisted of dimethylamine, the rest of formaldehyde (4). These results indicated that secondary oxidation of DMAP had occurred after covalent binding of the ring to hemoglobin.

In another experiment, we compared the pattern of radioactive tryptic peptides starting with either [^{14}C-*phenyl*]DMAP or [$^{14}CH_3$]DMAP. In both assays, 3 mM hemoglobin reacted with 1.5 mM DMAP in 0.2 M sodium phosphate, pH 7.4 at 37°, under oxygen at atmospheric pressure for 30 min. The procedure of chain separation, β-globin denaturation, and tryptic cleavage was carried out under carbon monoxide or nitrogen as described above. Digestion of the same amount of β-globin in both experiments yielded nearly equal quantities of the ring and methyl label in Peptides II and III (pooled Fractions II and III) which, after Raney nickel treatment, liberated 87% of [$^{14}CH_3$]DMAP and 83% of [^{14}C-*phenyl*]DMAP, respectively. In contrast, the $^{14}CH_3$ label of the higher molecular weight peptides was only one-fifth that of the ^{14}C-*phenyl* label. Thus, the higher molecular weight peptides with the ring label had lost extensively the dimethylamino group of DMAP. These findings support our hypothesis that transient quinonimine thioether formation was responsible for the cross-linking with the COOH terminus.

For steric reasons, the most probable candidate for cross-linking with the cysteinyl-thioether of DMAP was the COOH-terminal histidine, in agreement with the finding that one histidine in the hydrolyzed Peptide I was missing. Since hemoglobin modified by DMAP was probably frozen in its quaternary *R*-state which should influence the tetramer-dimer equilibrium, we tried to separate different hemoglobin species by molecular sieving.

Hemoglobin (3 mM Fe) was allowed to react with [^{14}C-*phenyl*]DMAP, 1.5 mM in 0.2 M sodium phosphate, pH 7.4 at 37°, under oxygen for 30 min. After reduction of ferrihemoglobin with sodium dithionite under nitrogen, the solution (250 mg of hemoglobin) was chromatographed on Sephadex G-100 fine (4 cm inner diameter × 200 cm) with 1 M $MgCl_2$ in 100 mM Tris-HCl (pH 7.0) under nitrogen. From the retention volume of untreated hemoglobin, the higher molecular weight Fraction I corresponded to tetrameric hemoglobin, whereas the lower molecular weight Fraction II was attributed to α,β-dimers. Both fractions, which exhibited nearly the same specific radioactivity, were flushed with carbon monoxide and treated with carboxypeptidase A. Figure 7 shows that fraction I released histidine in a manner similar to that of untreated hemoglobin, whereas Fraction II released only one-third that amount. When both fractions were examined spectroscopically in the Soret region,

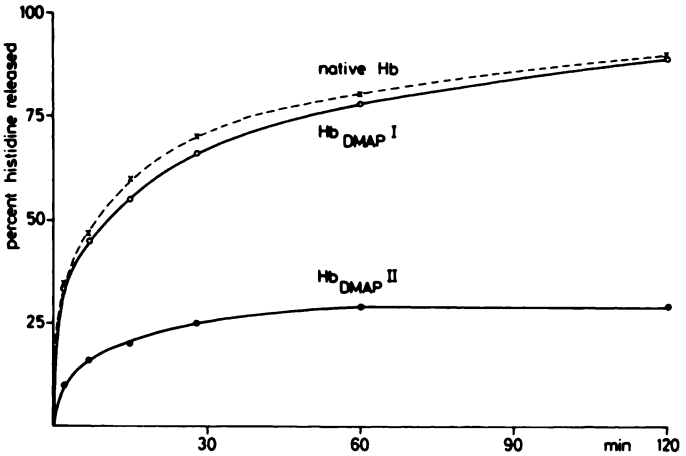

FIG. 7. *Release of histidine from hemoglobin by treatment with CPA*

Digestion was performed in 0.2 M sodium bicarbonate (pH 9.2) with 1:50 CPA (w/w) under carbon monoxide with native hemoglobin (×), Hb_{DMAP}I (○), and Hb_{DMAP}II (●) separated by the chromatography on Sephadex G-100 (100% = 0.5 histidine/Fe).

deoxyhemoglobin of Fraction I showed no change in absorbance upon addition of inositol hexaphosphate, whereas Fraction II showed an 8.5% increase in absorbance at 430 nm upon addition of inositol hexaphosphate.

DISCUSSION

Site and mechanism of covalent binding of DMAP to hemoglobin. The above experiments confirm cysteine 93β as the primary binding site in human hemoglobin for DMAP. Thus, no radioactivity was found in the isolated α-chains, and tryptic peptides from β-globin were isolated which exhibited the radioactive DMAP label and amino acids on an equimolar ratio corresponding to the peptides 83–95 β and 83–104β. These typical peptides containing cysteine 93β, which were found by tryptic digestion (24), liberated DMAP nearly quantitatively upon desulfuration with Raney nickel. Hence, similar to reactions with GSH (8), DMAP was presumably bound to the SH groups of cysteine 93β via thioether linkage. Quantitative blockade of these titratable SH groups by DMAP, and inhibition of that covalent binding by preceding treatment with pCMB or *N*-ethylmaleimide support this idea.

A major proportion of DMAP, which was bound to cysteine 93β, gave rise to additional changes in hemoglobin. Depending on oxygen pressure and time of incubation, DMAP reacted not only with this reactive SH group but secondarily with a histidine residue at the COOH terminus of the β-chain. This event caused intramolecular cross-linking and resulted in a tryptic peptide containing the residues 83–104β and 133–146β in a molar ratio to DMAP. On the basis of weight and radioactivity (assuming 1 mole of DMAP bound to this peptide) a molecular weight of 4170 was calculated, which agrees well with the theoretical value of 4120, the sum of the fragments 83–104β, 133–146β, and DMAP. Since amino acid analysis of this peptide indicated loss of one histidine, we assumed a linkage of DMAP between cysteine 93β and one histidine at the COOH terminus. Thereby a loop within the β-chain was formed from which trypsin split off the tryptic peptides 105–120β and 121–132β.

From the two possible histidine residues at the COOH terminus, 143β or 146β, we favored 146β to be cross-linked by DMAP. The COOH terminal histidine was shown by Perutz (25) to form a salt bridge with aspartate 94β, and hence comes in close contact with cysteine 93β. In addition, experiments with carboxypeptidase A showed that hemoglobin which had bound DMAP released only a small proportion of histidine and tyrosine on treatment with carboxypeptidase A. Thus, histidine 146β with its imidazole nitrogen, especially prone to electrophilic attack, was most probably cross-linked by DMAP with cysteine 93β.

Some light was thrown on the sequence of molecular events during covalent binding by comparison of the reactions of [$^{14}CH_3$]DMAP and [^{14}C-*phenyl*]DMAP. As already reported (4), DMAP readily autoxidizes with transient formation of *N,N*-dimethylquinonimine. This extremely labile intermediate, with an apparent half-life of seconds at pH 7.4 and 37°,[2] is rapidly hydrolyzed into *p*-benzoquinone and dimethylamine. Oxyhemoglobin greatly enhances the oxidation of DMAP and the liberation of dimethylamine. At 3 mM oxyhemoglobin, the inital rates of dimethylamine formation from DMAP was 100 times faster than in the absence of hemoglobin (4). Thus, as shown in Fig. 6, the early events in the reaction of DMAP with hemoglobin, i.e., covalent binding of the phenyl moiety and liberation of dimethylamine, proceeded at similar rates at 40, 140, and 680 mm Hg oxygen pressure. However, the secondary events showed a marked dependence on oxygen pressure. DMAP that has been bound to cysteine 93β via a thioether linkage is—for obvious steric reasons—no longer oxidized by oxyhemoglobin but by dissolved oxygen, presumably to yield a quinoid structure from which dimethylamine is slowly liberated. A strikingly similar behavior has been reported previously for glutathionyl thioethers of DMAP (8). It is probably this secondary event which gives rise to a quinoid thioether, which liberates dimethylamine and undergoes electrophilic attack to the imidazole nitrogen of histidine 146β. Hence, the cross-linked peptide bearing the phenyl moiety of DMAP was almost free of the dimethylamine label.

The scheme in Fig. 8 summarizes these reactions. Ferrihemoglobin formation catalyzed by DMAP (Reaction *1*) is terminated by covalent binding, presumably of the *N,N*-dimethylquinonimine to cysteine 93β in human hemoglobin via a thioether linkage (Reaction *2*). This primary event does not fundamentally alter the functional properties of hemoglobin. This unstable thioether

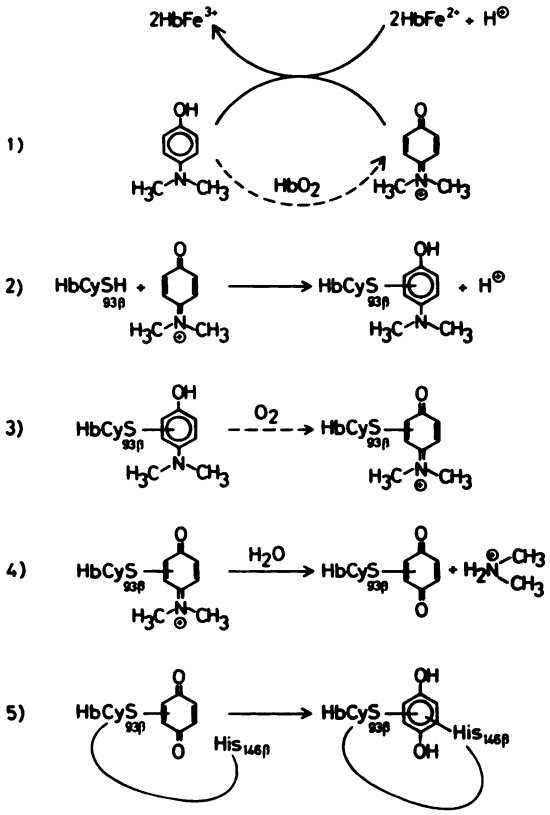

FIG. 8. *Tentative scheme of the reactions of DMAP with human hemoglobin*

[2] P. Eyer, unpublished data.

is secondarily oxidized by molecular oxygen, which gives rise to a quinoid structure (Reaction *3*) from which dimethylamine is hydrolytically released (Reaction *4*). Electrophilic attack of this quinoid thioether to the COOH-terminal histidine of the β-chains then leads to intramolecular cross-linkage (Reaction *5*). This "denaturation" reaction, which produces the changes in the quaternary structure of hemoglobin, provides the rationale as to the crucial alterations of the functional properties, because the "Perutz mechanism" (25) with the prerequisite of a freely movable COOH terminus of the β-chains no longer functions.

Consequences for the functional properties of hemoglobin. In order to describe the cooperativity of hemoglobin, a two-state model was formulated by Monod *et al.* (26) and confirmed by Perutz (25), who characterized two different structures of oxyhemoglobin and deoxyhemoglobin by X-ray crystallography. The oxyhemoglobin conformation was designated as *R*-state and the deoxyhemoglobin conformation as *T*-state. Today this model is most generally accepted, although it may be too simple for special cases [for a recent review, see Imai (27)]. Allosteric transition of the *T*-state, with low ligand affinity, to the *R*-state, with high ligand affinity, is coupled with the disruption of several constraining bonds. These constraints are mainly salt bridges, the disruption of which gives rise to extra protons, i.e., the alkaline Bohr effect. Forty per cent of these protons originate from the salt bridge between His 146β and Asp 94β. Organic phosphates, especially DPG and IHP, form several additional salt bridges between the β-chains, thereby constraining the $\beta_1\beta_2$ contact. As a result, the *T*-state of hemoglobin is favored (28). The two structures of hemoglobin exhibit distinct spectral properties. With ferrihemoglobin at neutral pH, Perutz *et al.* (29) reported marked spectral changes in the α- and β-bands of hemoglobin upon addition of IHP. These authors proposed that IHP changes the labile allosteric equilibrium and "switches" aquomethemoglobin from the predominant *R*-structure into the *T*-structure.

Hb_{DMAP} had an increased oxygen affinity which was about 10-fold higher than normal in 0.2 M sodium phosphate (pH 7.4). The cooperativity upon ligation expressed by the Hill coefficient, *n*, was decreased from about 3 to nearly unity. These changes were much less prominent with Hb_{PMB} or hemoglobins modified with other sulfhydryl reagents. When the oxygen affinities were examined at various pH values, the dependence of $pO_2 50$ on pH was weaker with Hb_{DMAP} than with native hemoglobin. Similarly, the release of protons upon ligation was only one-half that of native hemoglobin; i.e., the alkaline Bohr effect was reduced to about 50%. This value exactly fits the expected effect if one assumes that the salt bridge formation between His 146β and Asp 94β is hindered.

The unchanged oxygen affinity of Hb_{DMAP} upon addition of DPG was paralleled by the inability of deoxy-Hb_{DMAP} to bind DPG as revealed by gel chromatography. Under the same conditions, IHP carrying more negative charges remained bound. All of these results point indirectly to a largely prevailing *R*-state of Hb_{DMAP}, even in the absence of ligands. Examination of the spectral properties of Hb_{DMAP} showed that $HbFe^{3+}_{DMAP}$ was not "switched" into the *T*-state by IHP. This needed more drastic forces: when Hb_{DMAP} was deoxygenated, which normally alone induces *T*-structure formation, additional IHP had some effects. The same difference spectrum was also seen upon addition of IHP to *N*-succinimido-desArg-deoxyhemoglobin and hemoglobin Kempsey, as reported by Perutz *et al.* (30). It is worth noting that the height of the extinction difference at 430 nm was considerably lower with Hb_{DMAP} and amounted to only one-fourth that observed by the Cambridge group.

Finally, Hb_{DMAP} exhibited a different tetramer-dimer equilibrium. Because of the prevailing *R*-structure with its diminished constraints, deoxy Hb_{DMAP} dimerized to a significant extent, as shown by gel filtration studies. In addition, these experiments revealed that the Hb_{DMAP} preparation consisted of two different species, although both types bore the DMAP label. One type, with normal low dissociation, exhibited an obviously unchanged COOH terminus of the β-chains and showed normal digestibility by carboxypeptidase A. Moreover, deoxyhemoglobin of this type showed no spectral changes upon addition of IHP, indicating that this hemoglobin had already formed the quaternary *T*-state on deoxygenation. In contrast, the second type, which showed an increased dissociation into dimers, released only a small part of histidine on treatment with carboxypeptidase A and exhibited marked spectral changes upon addition of inositol hexaphosphate. The extent of these changes nearly reached the values of NES-des-Arg-hemoglobin and hemoglobin Kempsey as reported by Perutz *et al.* (30). Thus, this hemoglobin species was unable to form a normal quaternary *T*-state upon mere deoxygenation.

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