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Effect of aromatic isothiocyanates on the functional properties of human hemoglobin

Role of the stereochemistry of the charged group

Rodolfo Ippoliti [a], Douglas Currell [b], Eugenio Lendaro [a], Andrea Bellelli [a], Massimo Castagnola [c], Martino Bolognesi [d] and Maurizio Brunori [a]

[a] *Dipartimento di Scienze Biochimiche e Centro di Biologia Molecolare del C.N.R., Università La Sapienza, Roma, Italy,* [b] *Department of Chemistry, California State University, Los Angeles, CA, U.S.A.,* [c] *Istituto di Chimica, Università Cattolica del Sacro Cuore, Roma and* [d] *Dipartimento di Microbiologia e Genetica, Università di Pavia, Pavia, Italy*

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The effect of chemical modification of hemoglobin with six derivatives of benzene isothiocyanate has been studied. The negatively charged reagents (isothiocyanates of benzoic and benzenesulfonic acids) markedly inhibit the interaction of hemoglobin with allosteric effectors such as H^+, Cl^- and organic phosphates; the affinity for heme ligands in the absence of effectors is reduced but cooperativity is maintained, making these modified hemoglobins suitable models for a possible 'blood substitute'. The only uncharged reagent tested (isothiocyanate of benzenesulfonamide) increases the oxygen affinity of hemoglobin and affects only slightly the interaction with heterotropic ligands; its potential use as an antisickling drug is under study.

1. Introduction

The heterotropic interactions in hemoglobin have been studied thoroughly since the beginning of this century [1–4]. The thermodynamic basis of these allosteric phenomena has been lucidly analyzed by Wyman, who has developed during the last 50 years the linkage theory using hemoglobin as a prototype for regulatory interactions at the molecular level [5–8]. Wyman's approach to the problem has made use of a rigorous analysis of experimental thermodynamic data obtained on hemoglobin, using either natural mutants or chemically modified proteins. Over the same time span, the structural basis of cooperativity and the physical interpretation of allosteric interactions have been investigated successfully by Perutz [9–12] who was able to frame the essential features of the allosteric model [13] in terms of atomic interactions.

Within this conceptual and physical framework, experimental data on modified human hemoglobin have served the double purpose of testing ideas and models, as well as providing new materials of possible biotechnological value.

In this paper we report a comparison of the effect on human hemoglobin of a series of iso-

Correspondence address: R. Ippoliti, Dipartimento di Scienze Biochimiche, Università degli Studi di Roma 'La Sapienza', Ple Aldo Moro 5, 00185 Roma, Italy.

Abbreviations: 4-, 3-, 2-ICBS, 4-, 3-, 2-isothiocyanate benzenesulfonic acid, respectively; 4-, 3-ITCB, 4-, 3-carboxyphenyl isothiocyanate, respectively; 4-ICSA, 4-isothiocyanate benzenesulfonamide.

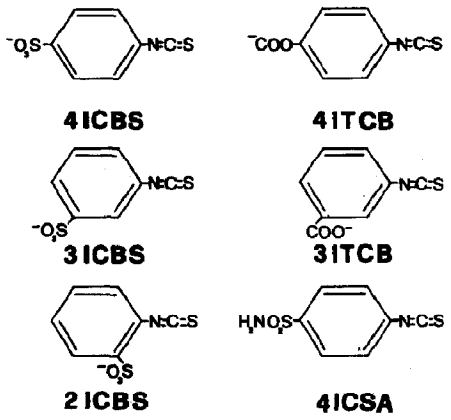

Fig. 1. Schematic representation of the reagents used.

meric molecules sharing as common features a benzene ring, an isothiocyanate group and a negatively charged substituent (carboxylate or sulfonate) whose position, with respect to the isothiocyanate group, is either *ortho, meta* or *para* (see fig. 1). As shown initially by Currell and coworkers [14,15], and extensively reinvestigated here, these compounds react covalently with the four amino termini of hemoglobin and yield a modified molecule with decreased oxygen affinity over a wide pH range above pH 7. It is shown that the presence of a negatively charged group is necessary to lead to a reduced oxygen affinity, as demonstrated by the modification of human hemoglobin with 4-ICSA [15] in which the sulfonate group is replaced by the uncharged sulfonamide group. Furthermore, modification of hemoglobin with some of these compounds apparently interferes with the oxygen-linked chloride-binding sites, since the oxygen affinity of the modified hemoglobin is independent of chloride concentration at pH 7; by contrast, the hemoglobin modified with 4-ICSA displays a normal response to chloride (see also refs 15 and 16).

A comprehensive investigation of the structure, equilibrium and kinetics of ligand binding, using six compounds to react with human hemoglobin, is reported below. The results are briefly discussed with reference to the possible use of these modified hemoglobin derivatives as a blood substitute [17].

2. Materials and methods

The compounds were synthesized as described by Dyson [18] and Maddy [19] or purchased from Transworld Chemicals (Washington, DC). Their chemical structures are shown in fig. 1.

The reaction with human hemoglobin stripped of organic phosphates was carried out as previously described [14,16].

Electrophoresis was carried out on 7.5% polyacrylamide gel in Tris-glycine buffer, pH 8.6 [20]. Electrophoresis of α- and β-chains was performed on cellulose acetate in Tris-glycine buffer (pH 8.6) containing 8 M urea: lyophilized samples of globin were dissolved in 8 M urea and run for 2 h at room temperature.

The site of the chemical modification was determined by HPLC of the polypeptide fragments obtained by 6 h tryptic digestion at pH 8 and 37°C. The column (Brownlee Lab RP 300) was equilibrated with 0.01 M acetic acid-ammonium acetate buffer (pH 6.04) and eluted with a gradient of acetonitrile (0–40%) in the same buffer. Chromatograms were recorded with a Perkin Elmer LC 85 analyzer at three wavelengths: 216, 254 and 280 nm.

Titration with *p*-chloromercuribenzoate according to the method of Boyer [21] indicated that the two β93 cysteine residues were unmodified.

The site of reaction is independent of the presence of heme ligands, as indicated by the identical functional properties of the modified hemoglobins obtained starting from either deoxy- or oxyhemoglobin.

Since these modified hemoglobins have not been crystallized as yet, construction of the three ICBS derivatives starting from the pyridoxal phosphate crystallographic coordinates of deoxyhemoglobin [22,23], was achieved by means of an interactive computer graphics program (FRODO, implemented on an Evans and Sutherland 3000-Digital VAX II/780 computer).

Oxygen-binding isotherms were recorded by means of the tonometric [24] or thin-layer dilution methods [25]. The kinetics of carbon monoxide combination were recorded by means of a Gibson Durrum stopped-flow apparatus or a flash photolysis instrument [26]. In the latter case, a stock

solution of each hemoglobin sample at a concentration of 10 μM/heme was equilibrated with 50 μM CO and a few grains of sodium dithionite were added when necessary. Stock solutions were used as such or serially diluted 1:2, 1:4, 1:8, 1:16 with CO-equilibrated degassed buffer. These were submitted to flash photolysis employing a 500 W lamp firing for 150 μs; the flash light was filtered where necessary by 1/2, 1/4 and 1/20. Data from both equilibrium and kinetic experiments were analyzed by means of a desktop computer (HP 87).

3. Results

3.1. Chemical characterization of modified hemoglobins

The reaction with all the compounds yielded homogeneous products corresponding to hemoglobin modified at the four amino termini (see below). In some cases, the reaction with 3-ICBS or 3-ITCB was found to give more than one electrophoretic species; in these instances, the modified hemoglobin was discarded and the reaction was repeated (without attempting to purify the various components).

Electrophoresis of the globin from modified hemoglobins (carried out in 8 M urea) showed only two protein bands, indicating that both the α- and β-chains reacted homogeneously. With the exception of 4-ICSA, the anodic mobility of the reacted chains was clearly increased as compared to the native chains.

Chemical analysis was carried out only with samples reacted with the three ICBS derivatives. The HPLC chromatogram of tryptic fragments from Hb 2-ICBS is reported in fig. 2 as an example of the results; assignment of the main peaks was made on the basis of the retention volumes, using trypsin-digested, unmodified human hemoglobin as a reference; the retention volume of the α_1 and β_1 fragments was clearly different from those of the reference HbA while those of all the other peaks were the same. Only in the case of Hb 3-ICBS did the attribution of the peaks prove difficult due to the incomplete digestion of the

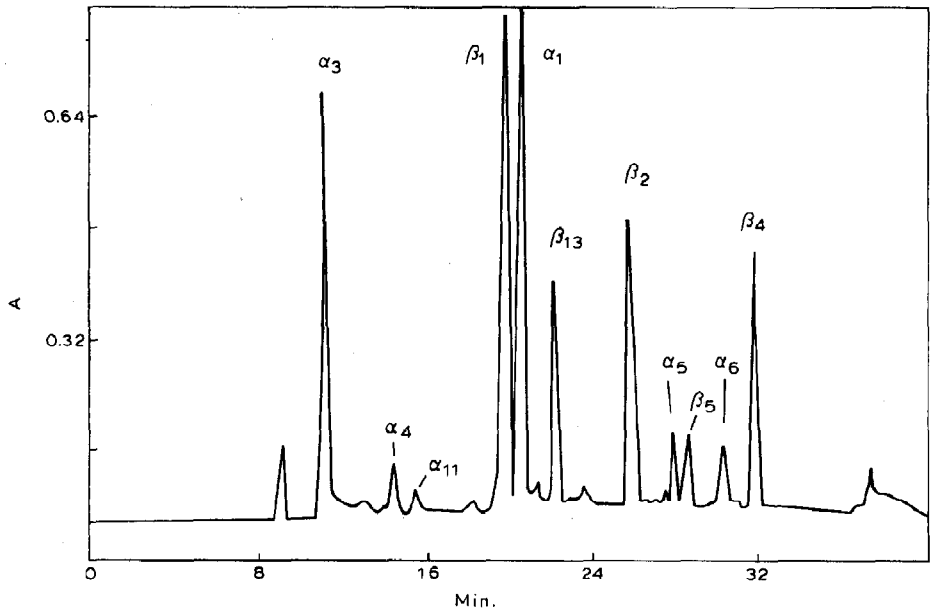

Fig. 2. HPL chromatograms of tryptic digestion of Hb 2-ICBS. Conditions are reported in the text. Absorbance was recorded at 254 nm.

Table 1

Oxygen-binding properties determined by thin-layer dilution and tonometric methods

Conditions: Tris or BisTris buffers + 0.1 M NaCl; $t = 20\,^{\circ}C$.

Sample	pH 8.4 [a]		pH 8.4		pH 7.2	
	log $p_{1/2}$	n	log $p_{1/2}$	n	log $p_{1/2}$	n
HbA stripped	0.48 [b]	2.8 [b]	0.05	2.3	0.52	2.2
Hb 4-ICBS	0.88	1.65	0.69	1.7	0.99	1.5
Hb 3-ICBS	0.49	2.4	0.43	1.8	0.96	1.9
Hb 2-ICBS	0.53	2.0	0.49	1.7	0.78	1.9
Hb 4-ICSA	0.12	2.0	0.08	1.6	0.68	1.6
Hb 4-ITCB	0.46	1.5	0.40	1.5	0.82	1.6
Hb 3-ITCB	0.48	2.0	0.52	1.6	0.85	1.5

[a] Thin-layer dilution method.
[b] Data at pH 8.0.

sample. The amino termini of the α- and β-chains were both fully modified, as demonstrated by the retention volumes in HPLC experiments, while the other peaks were substantially unaffected. These results show that ICBS reacts at the amino termini of both chains, irrespective of the stereochemistry of the sulfonate, in agreement with the initial results of Currell et al. [14,15].

To acquire information on the interactions of the charged sulfonate group with the residues of the DPG-binding crevice in between the two β-chains, analysis by computer graphics, using the atomic coordinates for oxyhemoglobin and deoxyhemoglobin [22,23], was carried out.

The two molecules of 4-ICBS are sufficiently far apart in the DPG-binding crevice (15–20 Å) not to give rise to steric hindrance, in agreement with the observed stoichiometry. The negatively charged sulfonate group of 4-ICBS bound to the N-terminal Val of one β-chain (β_1) may interact with the positively charged amino groups of Lys 144 and Lys 82 and/or with the imidazole group of His 143 of the opposite β-chain (β_2). Since these ionic interactions involve amino acid side chains of the contralateral β-chain, stabilization of the tetramer may be expected. This result was indeed observed by Currell et al. [27] for hemoglobin reacted with 4-ICBS, but was not confirmed by data obtained via flash photolysis (see below and ref. 16); this inconsistency is discussed below. In deoxygenated hemoglobin the Lys β 144 residue is distant from the N-termini of the contralateral β-chain (> 10 Å) and interacts with the Glu 90 side chain; therefore, this interaction is unlikely to happen. The other interactions of oxyhemoglobin (with His 143 and Lys 82) are maintained.

The charged group of 3-ICBS bound to the N-terminal residue of one β-chain can interact with Lys 144 and His 143 of the other β-chain, but not with Lys 82; furthermore, the 3-sulfonate is pulled towards the negatively charged carboxyl terminal of the contralateral β-chain.

The sulfonate group of 2-ICBS undergoes essentially the same interactions as 3-ICBS, even though its rotation in the cavity is severely limited.

On this basis, one might expect that differences between Hb 4-ICBS on the one hand, and Hb 2-ICBS and Hb 3-ICBS on the other, may be related to the lack of interaction of the latter compounds with Lys 82 across the dyad axis in the DPG-binding crevice among the two β-chains. Thus, in these cases a less stable tetramer may be expected as compared to Hb 4-ICBS.

3.2. Oxygen-binding properties

The oxygen-binding properties of hemoglobin reacted with all the compounds were investigated under the following conditions: pH 6.5–8.5, [2,3-DPG] = 0–50 mM, $t = 20\,^{\circ}C$ and $[Cl^-] = 5$ mM–1 M. The results for all modified hemoglobins are summarized in table 1; oxygen-binding isotherms obtained at pH 8.4 by means of the thin-layer

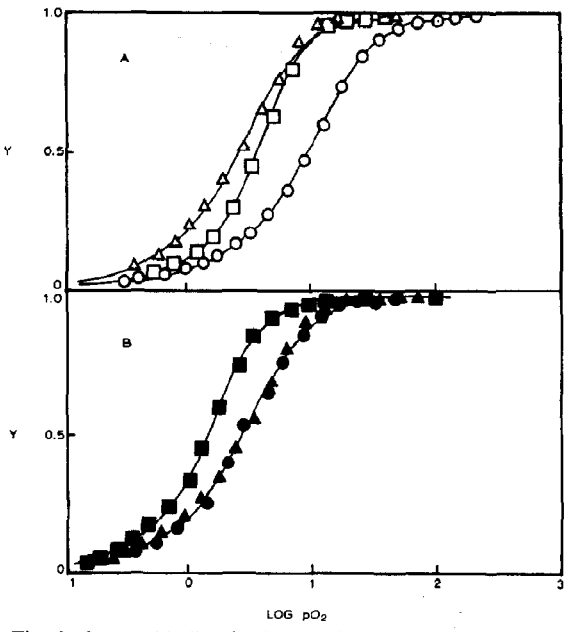

Fig. 3. Oxygen-binding isotherms obtained via the thin-layer dilution method in 0.1 M Tris + 0.1 M NaCl, pH 8.4; $t = 20\,^{\circ}C$. (○) Hb 4-ICBS, (△) Hb 3-ICBS, (□) Hb 2-ICBS, (■) Hb 4-ICSA, (●) Hb 4-ITCB, (▲) Hb 3-ITCB.

dilution method [25] were also analyzed using a least-squares nonlinear regression fitting program according to the Adair equation [28] and the two-state model [13] and some of the fits are shown in fig. 3. It is clear from the data that all the compounds tested reduce both the oxygen affinity and the cooperativity of hemoglobin, except for 4-ICSA; this implies that reduction in oxygen affinity requires a negatively charged group on the aromatic residue bound to the N terminal Val of the β-chains.

3.3. *Effect of heterotropic ligands*

The site of the chemical modification is such that binding of organic phosphates, Cl^- and H^+ should be affected. Thus, we investigated the effect of these heterotropic ligands on the functional properties of all the modified hemoglobins, and analyzed the results in the framework of linkage theory as developed by Wyman [5,6].

While Hb 4-ICSA is sensitive to Cl^- over the whole pH range explored [15], all the other modified hemoglobins (reacted with a compound carrying a charge) are completely insensitive to this effector in the alkaline pH region (figs 4 and 5A); a minor effect of Cl^- on the oxygen-binding properties was detected at pH < 7. This may be in-

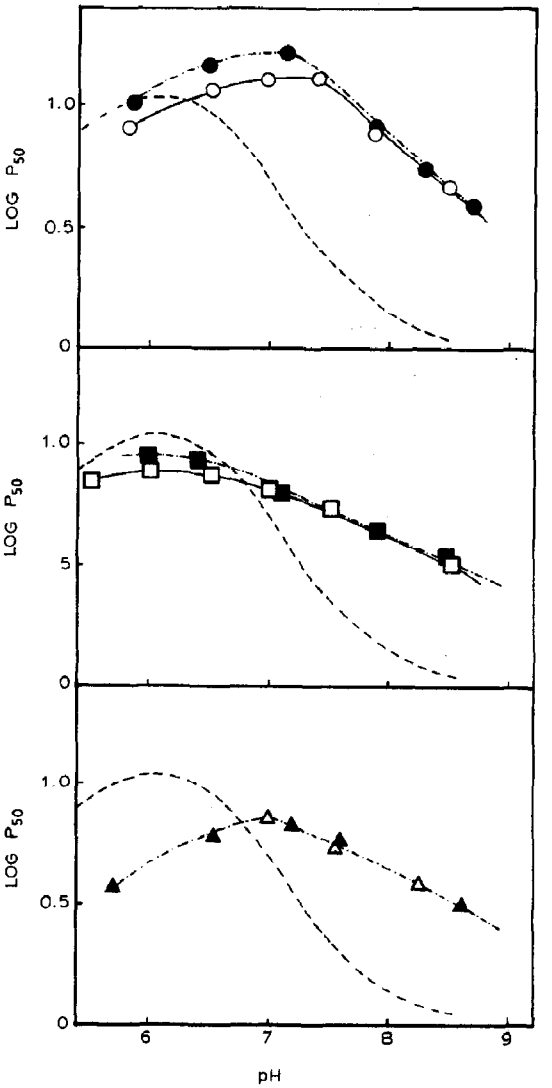

Fig. 4. Bohr effect for some reacted hemoglobins in the presence and absence of Cl^-. Buffers: 0.1 M Tris or BisTris in the absence or presence of 0.1 M NaCl; $t = 20\,^{\circ}C$. (○, □, △) Experiments in the absence of Cl^-; (●, ■, ▲) in the presence of 0.1 M NaCl; (○, ●) Hb 4-ICBS; (□, ■) Hb 2-ICBS; (△, ▲) Hb 3-ITCB. (------) Bohr effect of human hemoglobin in the presence of 0.1 M NaCl.

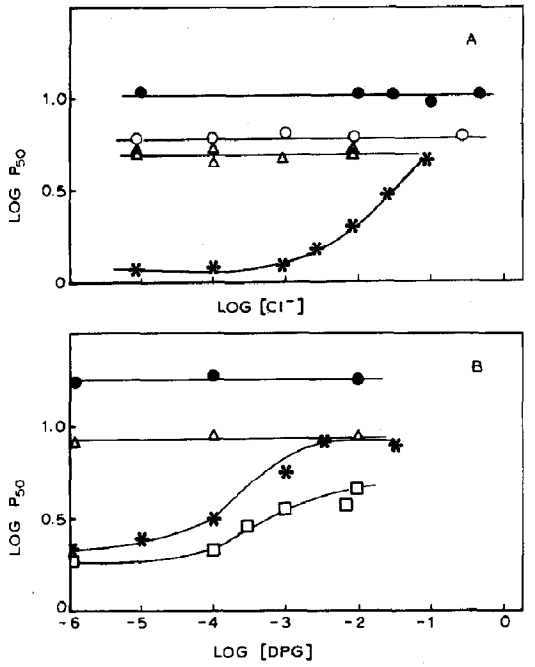

Fig. 5. (A) Effect of Cl^- at pH 7.3 in 0.1 M BisTris buffer. (B) Effect of DPG in 0.1 M BisTris buffer, pH 7.3; $t = 20\,°C$. (*) Hb A, (●) Hb 4-ICBS, (△) Hb 3-ITCB, (●) Hb 2-ICBS, (□) Hb 4-ICSA.

ferred from inspection of the results reported in fig. 4, where it is shown that the Cl^--dependent Bohr effect is absent, while Hb 4-ICBS and Hb 2-ICBS display some response to this effector at acid pH.

In fig. 4, the Bohr effect of Hb 3-ITCB, Hb 2-ICBS, and Hb 4-ICBS is compared with that of native human hemoglobin. The Bohr coefficient ($\Delta \log p_{1/2}/\Delta pH$) is markedly reduced in all modified hemoglobins; only Hb 4-ICSA displays a Bohr effect which is slightly reduced with respect to HbA (see table 1).

The effect of 2,3-DPG on oxygen affinity (fig. 5B) was found to be absent in all cases except for Hb 4-ICSA, as expected on the basis of the site of modification and the reduced or absent sensitivity to Cl^-.

3.4. Carbon monoxide combination kinetics and tetramer-dimer dissociation

Fig. 6 shows representative time courses of CO combination determined by stopped flow at pH 7.3, in the absence and presence of (3 mM) 2,3-DPG. In all cases, the combination is visibly autocatalytic, without any evidence for a quickly reacting species; thus, dissociation into $\alpha\beta$ dimers does not occur in the deoxygenated derivatives. In agreement with oxygen-binding data (see above), 2,3-DPG has a clear effect on the kinetics of CO

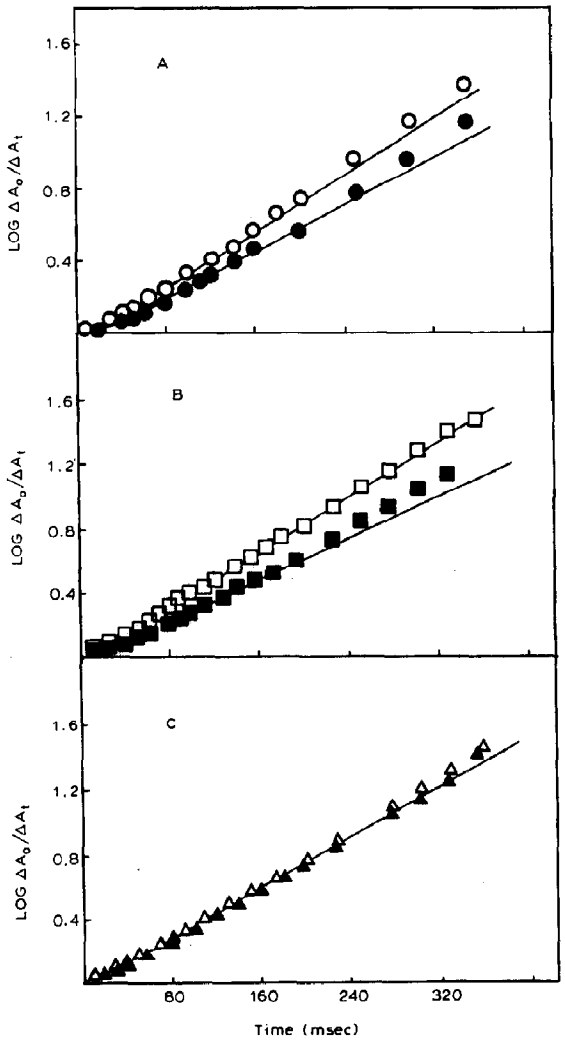

Fig. 6. CO combination kinetics by stopped flow in the presence and in the absence of 2,3-DPG. Concentrations: 4 μM for all hemoglobins, 2.8 mM for DPG and 100 μM for CO before mixing; $t = 20\,°C$. (○, □, △) Hemoglobin in the absence of 2,3-DPG; (●, ■, ▲) presence of 1.4 mM DPG; (○, ●) Hb A; (□, ■) Hb 4-ICSA; (△, ▲) Hb 3-ICBS.

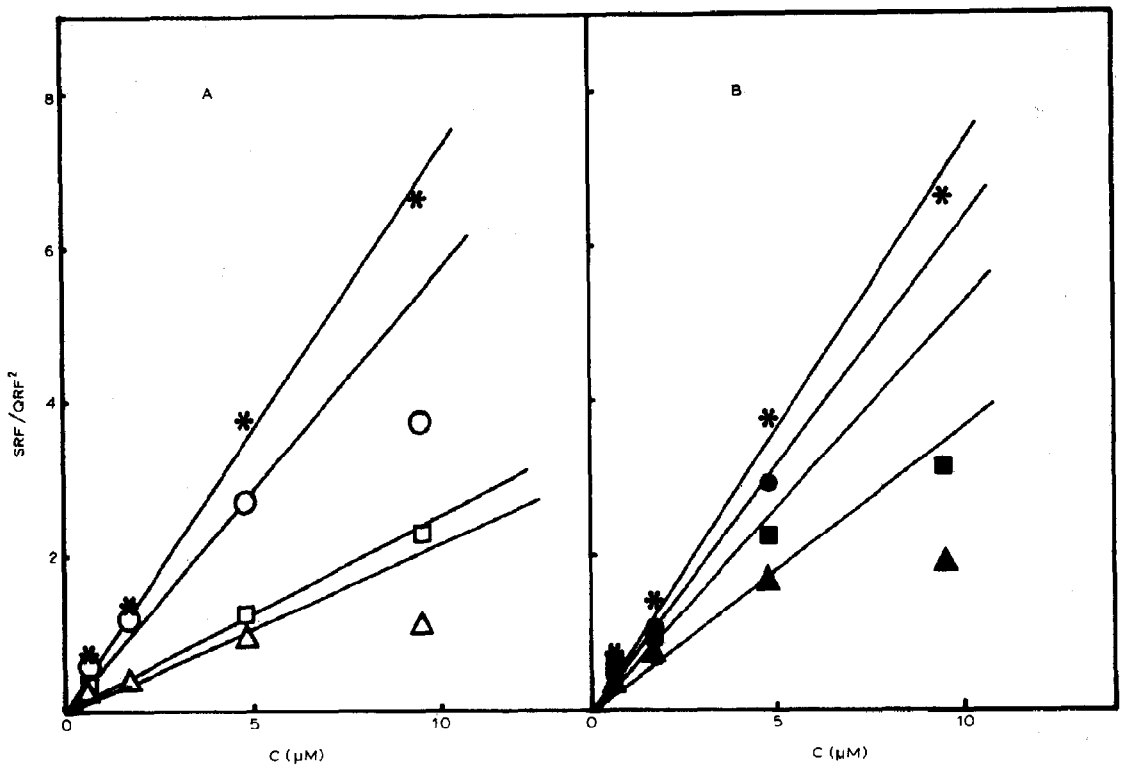

Fig. 7. Tetramer dimer dissociation of CO derivative of reacted hemoglobins, obtained by flash photolysis as a function of protein concentration. Conditions: 0.1 M BisTris buffer, pH 7.0, containing 0.1 M NaCl; $t = 20\,^{\circ}C$. (*) Hb A, (○) Hb 4-ICBS, (△) Hb 3-ICBS, (□) Hb 2-ICBS, (●) Hb 4-ITCB, (▲) Hb 3-ITCB, (■) Hb 4-ICSA.

combination of HbA and Hb 4-ICSA, while this effect is absent in the hemoglobins reacted with charged compounds (see fig. 6 for Hb 3-ICBS).

Table 2

Equilibrium constants for the tetramer-dimer dissociation of carbonmonoxyhemoglobin, calculated from flash photolysis data

Conditions: 0.1 M BisTris buffer + 0.1 M NaCl, pH 7.0; $t = 20\,^{\circ}C$.

Hemoglobin	K_d (μM)
Hb 4-ICBS	7.6
Hb 3-ICBS	19
Hb 2-ICBS	16
Hb 4-ITCB	6.6
Hb 3-ITCB	11
Hb 4-ICSA	8.2
Hb A	5.6

Flash photolysis experiments on the CO derivatives were carried out in order to measure the fractional dissociation of liganded tetramer into dimers, by determining the amount of quickly reacting form (QRF) at different protein concentrations [4,29–31]. This approach provides significant information, but may suffer from the difficulty of attributing all the observed QRF to $\alpha\beta$ dimers [32]. Complete (> 95%) photodissociation of the ligand was obtained for all sample concentrations except the highest (10 μM). Fig. 7 reports the results of these experiments in the form of the ratio between the fraction of slowly reacting form and the squared fraction of quickly reacting one; this corresponds to the reverse of the Ostwald law for the reaction $(\alpha\beta)_2 \leftrightarrows 2\alpha\beta$ and gives a straight line crossing the origin and with a slope 4-times the reciprocal of the dissociation constant. It can be seen that the experimental

value obtained at the highest protein concentration sometimes falls below the straight line (i.e., corresponds to more QRF than expected; these points were ignored for the calculation of the dissociation constant).

The tetramer-dimer equilibrium constants for all modified hemoglobins as well as HbA are reported in table 2. It may be seen that native hemoglobin, Hb 4-ICSA, Hb 4-ICBS and Hb 4-ITCB have similar low dissociation constants, while Hb reacted with 3-ICBS, 2-ICBS and 3-ITCB dissociates into dimers at somewhat higher concentration; the position of the charged group is clearly relevant in this respect, in good agreement with computer graphics analysis.

To exclude the possibility that sodium dithionite may affect the validity of the measurements [33], we carried out experiments at different dithionite concentrations starting from zero (obtained with a sample degassed and fully deoxygenated using ascorbic acid, ascorbic acid oxidase and catalase). We observed no effect of dithionite, since the amount of QRF was strictly the same in all cases.

4. Discussion

In this paper we have examined the functional properties of human hemoglobin modified with five aromatic isothiocyanates carrying a charge in the *ortho, meta* or *para* position and one uncharged compound. This has been undertaken in order to explore the effect of stereochemistry of the modifying reagent using qualitative predictions based on model building by computer graphics and functional data. In addition, it may be pointed out that reaction with these compounds may be of interest for medicine, since modified hemoglobins with reduced affinity and cooperative binding may be useful for engineering a possible blood substitute [17,34].

The oxygen-binding properties of the hemoglobins reacted with this series of isothiocyanate compounds are different from those of native HbA. In general, oxygen affinity and cooperativity are both reduced when a charged group is present in the modifying reagent, while the absence of a negative charge (as in Hb 4-ICSA) is associated with a small effect on the oxygen affinity and somewhat reduced cooperativity.

The alkaline Bohr effect is markedly reduced for all the reagents which carry a negative charge; this effect can be attributed mainly to binding of the reagent to the N termini of the α-chains, as demonstrated earlier with the hybrid hemoglobin tetramers modified only on the α- and β-chain [16]. Val 1α is a Cl^--dependent Bohr residue [9,12], whose pK is 7.0 in oxy-Hb and 7.8 in deoxy-Hb [35]; this residue is the site of reaction for isothiocyanate with formation of an unreactive diiminophenyl derivative ($Hb\text{-}NH_2 + R\text{-}N{=}C{=}S \rightarrow Hb\text{-}NH\text{-}CS\text{-}NH\text{-}R$). Thus, binding of H^+ and Cl^- is abolished, and an important Cl^--linked Bohr site is lost: the decrease in the alkaline Bohr effect can be partially attributed to this covalent modification. The reduced alkaline Bohr effect also produces an apparent increase in the acid Bohr effect and a shift to the right of the maximum of the log $p_{1/2}$ vs pH curve.

Let us consider the various Cl^--binding sites for HbA, Val 1 and Arg 141 on the α-chains and Lys 82 and His 146 on the β-chains.

His 146 β, a weak Cl^--binding site, also in view of the salt bridge formed in deoxy-Hb with Asp 94 [9,12], lies far apart from the site of chemical modification. We have no evidence for perturbation of this residue.

Lys β 82 is a weak chloride site [9,12] and is responsible for the competition of Cl^- with organic polyphosphates. According to the computer graphics analysis (see above), Lys 82 can interact with the charged sulfonate and carboxylate groups only when they are in position 4 of the modifying reagent; in fact, it is suggested that Lys 82 forms a salt bridge with 4-ITCB and 4-ICBS, thereby abolishing the effect of Cl^- [16].

Val 1α was discussed above; Arg 144α is quite close to the N-termini and may therefore interact with the negatively charged groups, with the resulting effect of suppression of Cl^- binding; the permanent interaction of the benzoate or sulfonate group with this residue is likely to mimic the allosteric effect of Cl^-, thereby reducing oxygen affinity.

In agreement with previous data [16], we can conclude that isothiocyanates of benzoic or benzenesulfonic acid modify H^+ uptake at pH > 7.0, and affect most (if not all) of the residues responsible for the alkaline Bohr effect on the α-chains.

The functional properties of the modified hemoglobins are insensitive to DPG, except for 4-ICSA as demonstrated by equilibrium and kinetic (stopped-flow) experiments; thus the binding site can still be accessible to DPG when the modifying reagent does not possess a charged residue (see, for example, figs 5 and 6). This finding is consistent with computer graphics analysis, which indicates that only two net positive charges (those of Lys β 82) are free to interact with the anions.

The results on the dissociation of HbCO tetramers into dimers conflict somewhat with previous results [27] and are difficult to interpret. Flash photolysis data indicate that dissociation of the modified hemoglobins is enhanced after specific modification of the DPG-binding site, as indicated by the dependence of the quickly reacting component on protein concentration. This is consistent with other observations reported on naturally glycosylated human hemoglobin [36], hemoglobin modified with glucose 6-phosphate [37] and pyridoxal phosphate (unpublished data, from this laboratory). However, it may be recalled that human hemoglobin reacted with 2-nor-2-formylpyridoxal phosphate [38], which cannot dissociate into dimers, displays a significant amount of QRF in flash photolysis experiments starting from HbCO, even though it reacts slowly in stopped-flow experiments [39]. This type of result is a clearcut example that correlation of the fraction of QRF with free dimers is not always straightforward. Examination of crystallographic data on Hb PLP [22], and computer graphics analysis on Hb 4-ICBS, Hb 3-ICBS and Hb 2-ICBS, indicate that favorable ionic interactions between the negatively charged group of the modifying reagent bound to one β-chain and positively charged residues of the contralateral β-chain are possible. This suggests a stabilization of the tetramer, as observed [27] in the case of the oxygen derivative of Hb 4-ICBS.

The flash photolysis data fail to show stabilization of the modified tetramer as compared to Hb A, but clearly indicate an influence of the stereochemistry of the reagent: when the negatively charged group is in position 4, the tetramer dimer equilibrium is hardly affected, while destabilization of the liganded tetramer is clearly detected when the substituent is in positions 3 and 2. Since the region of the amino-terminal residues of the α-chain in human hemoglobin is disordered and exposed to solvent, it is unlikely to contribute significantly to this effect; therefore, in the subsequent discussion, we refer only to the structural properties of the β-chain site. Computer graphics analysis indicates that a negative charge in position 4 forms three salt bridges with residues on the contralateral β-chain, while the same charge in position 2 or 3 is able to establish only two such interactions, since that with the Lys β82 is sterically impossible; furthermore, the proximity of the carboxyl terminal may actually result in net destabilization of the tetramer. This difference is clearly reflected in our data, which show a 3-fold increase in the dissociation constant between Hb 4-ICBS on the one hand and Hb 3-ICBS on the other.

We may offer only a tentative explanation for the inconsistency between photolysis and previous gel filtration data [27], based on the hypothesis that the reagent on the β-chain affects the dissociation properties of the T state (as reported [36] for glycosylated hemoglobin) as well as the stability of the T state. The equilibrium constant for dissociation into dimers of the T state, which is extremely low for unliganded hemoglobin, increases as ligand binding proceeds and ultimately exceeds that of the R state, whose affinity for the ligand is essentially the same as that of $\alpha\beta$ dimers [40]. In this respect, it is relevant that for Hb PLP, Hb 4-ICBS, Hb 3-ICBS, Hb 2-ICBS and Hb ICSA, the switch-over point [41] is higher than 3 (calculated from equilibrium data); thus, it is reasonable to assume that partially liganded T-state intermediates, populated in the flash experiments, may rapidly dissociate into dimers characterized by R-state reactivity.

These negatively charged compounds may have potential use as models for blood substitutes; in fact, the decrease in oxygen affinity and the maintenance of some cooperativity and Bohr effect are essential properties for this purpose, as

demonstrated by literature data [17]. The oxygen-binding properties of Hb 4-ICSA may in addition suggest possible applications in the treatment of sickle cell anemia: some preliminary data suggest that this molecule is able to traverse the red cell membrane, but more extensive studies are required in order to assess the clinical usefulness of these compounds.

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