INTERACTION OF HUMAN GLOBIN WITH AROMATIC AND ALICYCLIC HYDROCARBONS

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The presence of aromatic and alicyclic hydrocarbons increases the resistance of globin in a neutral medium to heat denaturation while on hemoglobin it has an opposite effect. The finding can be accounted for by assuming a saturation of hydrophobic regions of the molecule exposed when the heme group is severed. The stabilizing effect of hydrocarbons depends on their structure.

The conformation of human globin differs markedly from that of hemoglobin. While the hemoglobin molecule is a tetrameric oligomer of chain composition $\alpha_2\beta_2$, its structure being by 75–80% an α-helix, the globin molecule is an αβ-dimer with some 50% content of α-helix[1]. The hemoglobin tetramer is markedly stabilized by heme-globin interactions; it is assumed that when these are interrupted after splitting off the heme groups, the conformation of the corresponding contacts between subunits collapses, the hydrophobic core of the molecule is revealed and it dissociates into two dimeric globin molecules[1].

On the assumption of correctness of this concept of the globin molecular conformation, the stability of globin, reflected *e.g.* in its resistance to heat denaturation, should be favourably affected by the presence of low-molecular compounds of hydrophobic nature that would interact with the above-mentioned hydrophobic regions and that would make it more difficult for the globin molecules to associate spontaneously. The aim of the present communication was to verify this view by studying the effects of aromatic and alicyclic hydrocarbons.

The binding of various alkanes, aromatic and alicyclic hydrocarbons, to molecules of proteins in solution has been demonstrated by a number of authors[2–11]. Its extent was heavily dependent on the structure of the hydrocarbons as well as on the nature of the protein studied. The binding of hydrocarbons and other hydrophobic substances to globin has not been studied very extensively. Only Stryer[2] found that the removal of heme permits one molecule of 1-anilino-8-naphthalenesulfonate to be bound with its hydrophobic part to each globin chain, the degree of helicity of the protein remaining unchanged. However, he did not study the effect of this binding on globin stability. Hsu and Woody[12] later extended these studies to other naphthalene derivatives and to pyrenebutyric acid.

On the other hand, the binding of hydrocarbons to hemoglobin and myoglobin has been studied in considerable detail. Thus, Wishnia[3,4] analyzed thermodynamically the binding of a number

of alkanes to different forms of hemoglobin and myoglobin. He found the ratios of binding constants for the first molecule for different hydrocarbons and a given protein to be related to the ratios calculated from the distribution of the hydrocarbons between water and dodecylsulfate micelles. Kiehs and coworkers[5] found the stability of a number of more or less neutral nonionic aromatic structures to be linearly related to their distribution coefficient between 1-octanol and water. In the case of myoglobin no binding was observed and hence it was assumed that hemoglobin accumulates these substances at the interface between the four subunits of its molecule. Finally, Cann[6–8] found that aromatic compounds like benzene, iodobenzene and chlorpromazine are bound to the porphyrin ring of myoglobin heme, thus breaking its π-bond interactions with aromatic rings of the phenylalanine residues.

EXPERIMENTAL

Hemoglobin was prepared from fresh human erythrocytes by hemolysis with 3 volumes of water, followed by adsorption of the ghosts to a suspension of aluminium hydroxide and dialysis against 0·05M phosphate buffer of pH 7·0. "Native globin" was obtained by repeated precipitation in a neutral medium[13] and transferred by dialysis into 0·05M phosphate buffer of pH 7·0. Concentration of globin and hemoglobin solutions was estimated from the dry weight obtained by evaporation of 5 ml solution at 105°C. The aromatic and alicyclic hydrocarbons used for interaction experiments were freshly distilled.

Determination of heat stability of globin was carried out by combining 7 ml globin solution (the concentration varied from one experiment to the other between 0.95 and 1.47%) with 0.5 ml hydrocarbon and the mixture was heated for 3 h to 38°C. After cooling to 4°C, the solutions were centrifuged (15 min at 5 500 r.p.m.) and the protein concentration was estimated in the supernatant. The conditions were found as most suitable for assesseng the stabilizing effect of hydrocarbons since the differences between globin concentration in the supernatant in the presence of hydrocarbons and without them were largest. The effect of benzene on CO-hemoglobin and CN-methemoglobin was assessed on the basis of hemoglobin remaining in the supernatant after heating to 70°C for 1 h with and without hydrocarbon.

The CD *spectra of globin* and of globin solutions saturated with benzene, cyclopentane or cyclohexane were recorded on a JASCO ORD/UV-5 spectrophotometer equipped with CD accessory, in the region of 300–205 nm at 20°C in a 0·1 cm cuvette and with a protein concentration of 0·05%. The data are not corrected for the refraction index of the solvent; the values of mean residual ellipticity are shown in deg . cm^2 . $dmol^{-1}$.

RESULTS

When heated above 20°C, human globin tends to precipitate; a typical precipitation curve, representing the dependence of globin amount remaining in the supernatant after heating 1% solution for 1 h to 20–60°C is shown in Fig. 1. The temperature of half-precipitation, corresponding to the inflexion point of the curve, is about 38°C. If the globin solution has been saturated with benzene prior to heating, the half-precipitation temperature rose to 46°C, the stabilizing effect of benzene being apparent throughout the region of 20–60°C. The increased resistance of globin to denaturation by heat in the presence of benzene depends on the amount added. Fig. 2 shows this

for a 3-h heating to 38°C. The limiting stabilization value is reached at a molar ratio greater than 120 : 1 (benzene–globin).

On the basis of results obtained when following the interaction of globin with benzene it was possible to compare the effect of a series of aromatic and alicyclic hydrocarbons on the heat stability of globin. For comparison, we used the % amount of globin remaining in the supernatant after heating globin solutions saturated with the corresponding hydrocarbon for 3 h to 38°C. The results are summarized in Table I. The stability of the starting native globin varied in dependence on the quality of the preparation so that under the above conditions in the absence of hydrocarbons 35 to 48% of the original amount of protein remained in the supernatant. On the contrary, in the presence of hydrocarbons, relatively constant values were obtained irrespective of the globin preparation used.

To establish whether the stabilizing effect of hydrocarbons is not due to their effect on the secondary structure of the globin molecule, we measured CD spectra in the region of peptide absorption bands. Solutions of human globin saturated with benzene, cyclopentane or cyclohexane exhibit the same spectrum with minima at 222 and 208 nm, characteristic for the α-helical conformation of the molecule in the ab-

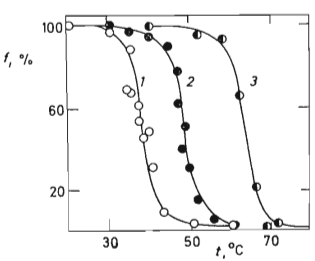

FIG. 1

Precipitation Curves of Human Globin (*1*), Globin in Benzene-saturated Solution (*2*) and of Human Methemoglobin (*3*)

Dependence of the protein fraction remaining in the supernatant after heating for 1 h, f (%), on temperature t.

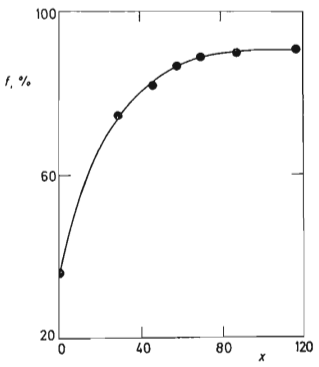

FIG. 2

Dependence of the Globin Fraction Remaining in the Supernatant after Heating for 3 h to 38°, f (%), on the Molar Ratio of Benzene to Globin (x).

sence of hydrocarbons. The mean residual ellipticity at 222 nm was −19400 for globin and −18950 for benzene-saturated globin, corresponding in both cases to about 50% content of the α-helical conformation[14]. Likewise, saturation with other hydrocarbons brought about no significant difference in the values of mean residual ellipticity as compared with native globin.

To check the assumption that the stabilizing effect of hydrocarbons on the globin molecule is due to their interaction with the hydrophobic regions exposed by separation of the heme, the effect of benzene on hemoglobin was examined. In view of the fact that the half-precipitation temperature is much higher with hemoglobin than with globin (Fig. 1) its solutions were heated for 1 h to 70°C. Two forms of human hemoglobin were used, carbonmonoxyhemoglobin (HbCO) and cyanomethemoglobin (Hb^+CN). The results in the following table refer to the amount of hemoglobin remaining in a solution of 0·05M phosphate buffer of pH 7 after heating for 1 h to 70°C with (f_b) and without (f_a) benzene.

Protein	f_a %	f_b %
HbCO	99	62
Hb^+CN	27	0

It may be seen that with both forms of hemoglobin the presence of benzene actually decreases the heat stability, resulting in a ready heat denaturation of the hemoglobin molecules.

DISCUSSION

The resistance of human globin to heat denaturation is markedly affected by the presence of some low-molecular weight compounds of hydrophobic nature, such as aromatic or alicyclic hydrocarbons. Of the compounds studied, the most effective were benzene and toluene, while *o*- and *m*-xylene were ineffective. The rise of heat stability of globin through the action of hydrocarbons can be explained as being due to their interaction with hydrophobic regions of protein molecules, exposed by splitting off the heme. Saturation of these regions decreases the tendency of the globin molecules to aggregate. It follows from the chiroptical properties that the secondary structure of the globin molecule does not change with hydrocarbon binding.

Even if the hydrocarbons studied generally decreased the tendency of globin molecules to aggregate, in no case was their effect comparable with that of heme. This provides further evidence that the stability optimum is brought about by the existence of a tetrameric molecule of hemoglobin which requires a chemical structure which fits precisely into the hydrophobic interior of the individual subunits, such as the very flat and highly conjugated protoporphyrin molecule.

TABLE I

Fraction of Human Globin in % (f) that Remains in Solution of 0·05M Phosphate Buffer of pH 7 after 3 h of Heating to 38°C in the Presence of Hydrocarbons

μ, Dipole moment of the hydrocarbon (from the Handbuch der organischen Chemie); R, molar refraction (calculated from the constants published in Lange's Handbook of Chemistry). The value of f for globin alone was 35—48%, depending on the preparation used.

Hydrocarbon	f, %	μ, D	R, cm^3
Cyclopentane	89	0·00	23·1509
Cyclopentene	63	0·90	22·3684
Cyclohexane	75	0·00	27·6896
Cyclohexene	65	0·60	27·0662
1,3-Cyclohexadiene	68	0·38	26·7577
Methylcyclohexane	59	0·00	32·5214
Benzene	95	0·00	26·1877
Toluene	84	0·34	31·0959
Styrene	64	0·31	36.4525
Isopropylbenzene	64	0.43	40·4245
tert-Butylbenzene	51	0·45	44·9567
Cyclohexylbenzene	37	—	52·2121
o-Xylene	36	0·60	35·8082
m-Xylene	46	0·40	35·9675
p-Xylene	72	0·00	36·0065

In the case of hemoglobin the presence of benzene actually decreased the heat stability. This fits in with the view of Cann[7,8] that the hydrocarbon disturbs the bond between heme and protein by shifting the charges, as well as by hydrophobic interactions.

When comparing the stabilizing effects of the various hydrocarbons one can discern a dependence on their structure. With all the four groups, group of cyclopentane, cyclohexane, mono- and disubstituted benzene, it follows that by decreasing molecular symmetry, whether due to unsaturation of bonds or of the substituent, the stabilizing effect on globin is reduced. The relationship is particularly expressed in the aromatic series where the monosubstituted benzene derivatives lose their stabilizing effect with the size of the substituent, while with xylenes it decreases with substituent asymmetry. This agrees with the satisfactory correlation between the stabilizing effect of hydrocarbon with the magnitude of the dipole moment or with molar refraction, the only exception being the cyclohexane group. The symmetry of the molecule is apparently not the only factor which bears on the effect of a hydrocarbon on the sta-

bility of globin molecules. If this effectivity is compared with the extent of hydrocarbon binding to serum albumin and β-lactoglobulin as studied by Mohammedzadeh and coworkers[11] it may be seen that a fine relation exists in the case of benzene, toluene, *p*-xylene, cyclohexane and methylcyclohexane. A lower effect is found with cyclohexenes and cyclohexadiene, as well as with *o*- and *m*-xylene. While there was no difference between the xylene isomers, their stabilizing effect decreases with the magnitude of the dipole moment.

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