Proton Nuclear Magnetic Resonance Study of the Association of Monovalent and Divalent Alcohols with Bovine Serum Albumin†

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ABSTRACT: Proton nuclear magnetic resonance (NMR) was used to study the interaction of bovine serum albumin (BSA) with alcohols having straight and branched chains and also with ethylene and propylene glycols. NMR spectra of the alcohols were monitored in D_2O solutions at a resonance frequency of 60 MHz when the albumin concentration was increased from 0 to 10% (w/v). The alcohol concentration, 3% (v/v), was kept low enough to avoid general conformational destabilization of the protein. Marked and selective broadening of the NMR lines of the various apolar parts of the alcohols was observed. The results were interpreted in terms of weak hydrophobic complex formation between alcohol molecules and apolar amino acid side chains of the albumin. It was found that the effectiveness of hydrophobic association between alcohols and BSA increases with increased length of the alcohol aliphatic chain and roughly follows the effectiveness of the alcohols at denaturing of the proteins, as known from spectroscopic measurements. The quantitative pattern of relative line broadening suggests that the interaction with albumin selectively immobilizes the methylene groups in primary alcohols and the methine groups in secondary alcohols. Also, the strength of association with BSA observed for straight-chain alcohols was markedly reduced for the secondary, tertiary, and chain-branched alcohols and also for the glycols. The above findings lead to a structural model of the alcohol–protein association. The hydrophobic complexes or interactions between alcohol and protein seem to be stabilized by the hydrogen bonds involving the hydroxyl groups of the alcohols and the peptide groups of the protein. The model incorporates features of several alternate mechanisms previously suggested for alcohol action.

The destabilizing effect of the aliphatic alcohols on the structures of a variety of proteins is both well established (Schrier et al., 1965; Herskovits & Jaillet, 1969; Russell & Cooper, 1969; Herskovits et al., 1970; Cassatt & Steinhardt, 1971; Tan & Lovrien, 1972; Brunori et al., 1972; Parodi et al., 1973; Russell, 1973; Ohama et al., 1973, 1977; Jacobson & Krueger, 1975; Brill et al., 1976) and well reviewed (Tanford, 1968; Eagland, 1975; Franks & Eagland, 1975). With regard to protein unfolding and denaturation, an increase in the length of hydrocarbon chain increases the effectiveness of the alcohols and branching decreases it.

The majority of workers attribute the protein unfolding caused by alcohols to perturbation of the hydrophobic bonds implicated in intramolecular protein stabilization, i.e., to nonspecific hydrophobic interactions between alkyl chains of the alcohols and apolar side chains of the protein. The satisfactory agreement (Herskovits et al., 1970) between the effectiveness of alcohols as protein denaturants and the binding constants evaluated using the classical theory of hydrophobic bonding (Nemethy & Scheraga, 1962) was essential for this interpretation. However, in contrast to the considerable amount of information about alternation of the protein structure by alcoholic denaturants, no direct experimental evidence has been available on the existence of protein–alcohol complexes. Quite recently Brill et al. (1976) found that the heme moiety of ferrihemoglobin and ferrimyoglobin can combine with methanol and ethanol to form stable complexes with dissociation constants on the order of 40–200 mM.

The polar end of the alcohol was usually considered to retain its hydrogen bonding to water, and its contribution to the free energy of binding alcohol–proteins was often neglected. Nevertheless, several mechanisms of alcohol action differing from simple hydrophobic interaction were also occasionally suggested for various proteins; for example, the model proposed for collagen (Russell & Cooper, 1969) involving solvent hydrogen-bonding interaction with the peptide bonds rather than the hydrophobic complexes with the side chains was an alternative.

With regard to the controversy concerning the mechanism of alcohol–protein interaction [for a review, see Franks & Eagland (1975)], the exchange kinetics studies of Woodward et al. (1975) should be emphasized. The effects of ethanol, ethylene glycol, and other cosolvents upon the hydrogen-exchange rates of some proteins investigated by these authors showed no evidence of protein–alcohol clathrate or strong complex formation at the low alcohol concentration proposed by Brandts (1969).

It seems that in order to demonstrate and specify in detail the structure of the complexes which may be formed between alcohols and proteins, it is necessary to apply techniques different from those conventionally used for the study of ligand binding and from those related to the overall properties of the perturbed macromolecule.

The present study was therefore undertaken to demonstrate the binding of alcohol to the globular protein bovine serum albumin by NMR spectroscopy. Our approach was based on the concept that the mode of binding to protein should find a reflection in the NMR spectra of the denaturant itself. The NMR line widths of the individual fragments of the molecules, sensitive to the degree and kinds of molecular motion in the sample, were expected in our case to distinguish between various mechanisms of immobilization of the alcohol molecules during their attack on the protein structure. Preliminary results showed marked and selective broadening of the NMR lines of alcohols in the presence of serum albumins, both bovine (BSA) and human (HSA) (Lubas et al., 1977a,b).

BSA was chosen for several reasons, but mainly because of its remarkable binding capacity for all kinds of ligands, well recognized also by NMR (Fisher & Jardetzky, 1965; Jar-

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Table I: Ranges of Alcohol/BSA Molar Ratio and Positions of the NMR Lines Analyzed for Individual Alcohols in the Alcohol-BSA-D_2O Systems

alcohol	positions[a] of individual signals[b] for line width analysis			order of alcohol/ BSA molar ratio[c]
	CH_3	CH_2	CH	
methanol	3.40 (s)			~500-2500
ethanol	1.26 (t)	3.66 (q)		~350-1750
1-propanol	1.03 (t)	1.53 (m) 3.74 (t)		~270-1350
2-propanol	1.20 (d)		4.05 (m)	~270-1350
1-butanol	0.88 (t)	3.62 (t)		~550-4450[d]
2-butanol	0.95 (t) 1.17 (d)	1.43 (m)	4.20 (m)	~550-4450[d]
2-methylpropanol	0.980 (d)	3.45 (d)		~220-1100
2-methyl-2-propanol	1.24 (s)			~210-1080
ethanediol		3.67 (s)		~370-1830
1,2-propanediol	1.07 (d)	3.53 (s) 3.40 (d)		~280-1400
1,3-propanediol		1.77 (m) 3.68 (t)		~280-1400

[a] Chemical shifts are in parts per million (±0.02 ppm) from external Me_4Si, pH 7.0. [b] From singlets (s), doublets (d), triplets (t), quartets (q), and multiplets (m), respectively. [c] Unless otherwise indicated, in the BSA range 2-10% w/v the alcohol concentration was 3% v/v. [d] In the BSA range 0.5-4% w/v, the alcohol concentration was 3% v/v.

detzky & Wade-Jardetzky, 1965; Zia et al., 1971; Fung & Sarney, 1971). Furthermore, the three-dimensional structure of BSA, forming hydrophobic grooves that can easily accommodate at binding several apolar molecules, has quite recently been determined (Brown, 1975).

The NMR data reported in this paper may lead to progress toward a detailed model of the association between alcohols and protein by allowing a clear correlation of various interpretations as to the mechanism of alcohol activity. Our model indicates cooperation between hydrophobic and hydrogen-bonding factors in favoring the protein denaturation events.

Materials and Methods

Five times crystallized and lyophilized bovine serum albumin (BSA) from Biomed (Cracow, Poland), lot no. 7/12/74, 9/19/75, and 20/2/75, was employed in all the experiments. The stock BSA solutions, usually in series 1–20% w/v, were freshly prepared by dissolving protein in D_2O without the use of a buffer. They were kept at 0 °C and always used for sample preparation within 1 day.

Concentration of BSA solutions was based on a direct weighting. The extinction coefficient of BSA was determined at 279 nm by the use of a Cary 118C spectrophotometer, and at a concentration range of 1–5 mg/mL it gave values which correspond to an $E_{1\%}^{1cm}$ (absorbance of a 1% w/v solution per cm) of 6.43 ± 0.05. Polyacrylamide gel electrophoresis (7.5% gel in Tris–EDTA–boric acid buffer, pH 8.4) shows typical profiles for high molecular BSA fractions with adequate purity. Ten percent of the protein existed in dimer form, as was estimated by column chromatography using Sephadex G-200 (Pharmacie, Sweden).

Reagent grade monovalent alcohols and glycols were purchased from POCh (Gliwice, Poland), except for 2-methyl-2-propanol (Reanal, Budapest, Hungary) and 2-butanol (VEB Berlin-Chemie, GDR). D_2O was purchased from the Institute of Nuclear Research (Swierk, Poland) and had a 99.8% isotopic purity.

The samples, usually 0.6 mL in volume, were prepared just before the NMR measurements by stirring the alcohol–D_2O mixtures and BSA solutions to the final desired concentration. Concentration of BSA and alcohol in the samples was expressed in percent w/v and v/v, respectively. For the molar concentration calculation, the densities of the respective alcohols (*Handbook of Physical Chemistry*, 1974) and the molecular weight of 66000 for BSA (Peters, 1975) were used.

Neutral pH was planned for all experiments. When necessary, adjustments were made under the control of an N-515 Mera-Elmed (Poland) pH meter by negligible volumes of 0.02 M NaOD or 0.02 M DCl prepared from 40% NaOD or 20% DCl in D_2O (Bio-Rad Laboratories, Richmond, CA). The reported pH values are the direct meter readings not corrected for the deuterium isotope effect.

Proton NMR spectra of alcohols in the studied systems were recorded at a resonance frequency of 60 MHz on a Varian EM-360 spectrometer, with tetramethylsilane (Me_4Si) in $CDCl_3$ as an external standard. A sweep rate of 600 Hz was used for all completed spectra analyzed here. The probe temperature remained constant at 25 °C throughout the experiments.

The NMR line widths ($\Delta\nu_{1/2}$) were measured at half-heights on the spectra expanded in the respective ranges with a scan width of 60 Hz/30 cm or 120 Hz/30 cm.

The line width was analyzed for singlets and for the chosen lines of the doublets, triplets, and occasionally higher multiplets, always in the range of BSA concentration where the multiplets were not completely fused and their components were easy to distinguish. The choice of the analyzed line in multiplets was based on the intensity and Lorentzian shape of the absorption curves.

The samples were independently prepared at least 5 times, and for each sample five independent spectrum registrations were carried out for line width measurements. The reported values of $\Delta\nu_{1/2}$ for the various proton groups are therefore the mean results of at least 25 separate measurements of line width.

Results

NMR Line Width Data for Monovalent Alcohols. A typical pattern illustrating the influence of bovine serum albumin on the NMR spectrum of the alcoholic denaturant is shown in Figure 1. With increasing BSA concentration a gradual broadening of the lines assigned to different aliphatic parts of the alcohol chain can be observed in the spectrum of 1-propanol. Repeated attempts failed to detect with increasing BSA concentration any changes in either the positions or the coupling constants of the line sets of the alcohols.

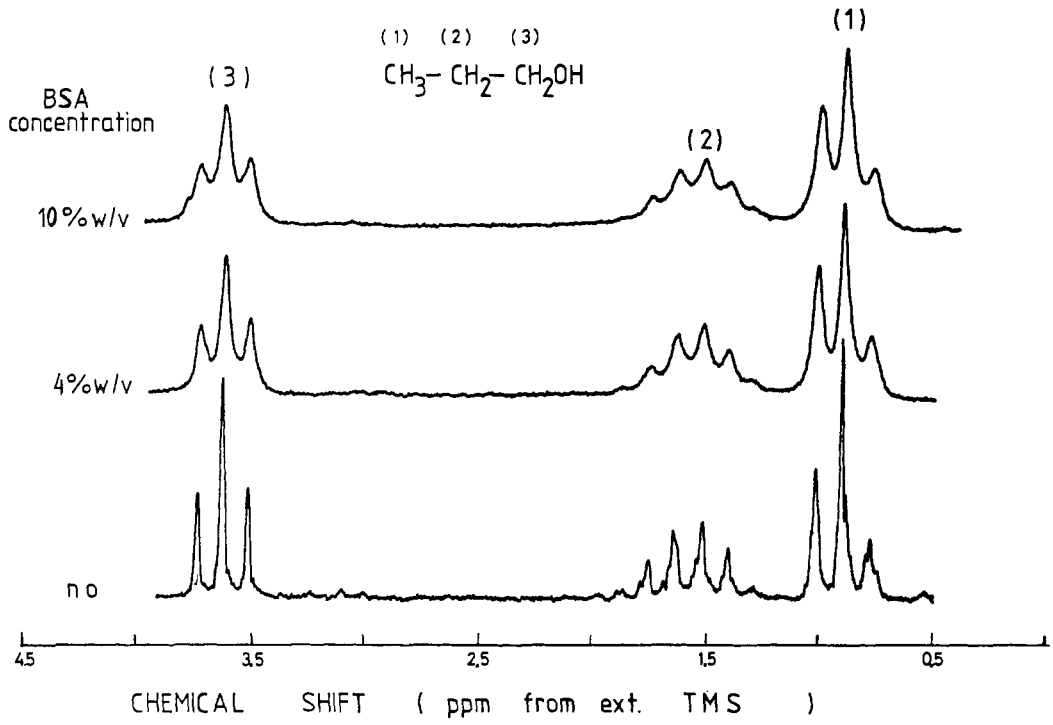

FIGURE 1: NMR spectra illustrating the effect of bovine serum albumin (BSA) on the proton groups of 1-propanol in D_2O; alcohol concentration 3% v/v, pH 7.0.

Detailed analysis of the width was carried out for alcohol lines chosen as indicated in Table I.

The results of line width measurements for methanol, ethanol, and two isomeric propanols are shown in parts A–D of Figure 2, respectively. The quantitative pattern of the line broadening reflects a strong dependence of the increase in NMR line width on the length of the hydrocarbon part of the alcohols and a higher increase in the line width for alcohols with longer alkyl chains.

Simultaneous comparison of data for the two isomeric propanols gives the essential feature of the reduced increase in line broadening for the alcohol with branched molecules. Also, different hydrophobic parts of the alcohol molecules seem to be subject to the line width increase to a varying extent. The last effect is especially readable for 1-propanol, where the lines assigned to the methylene group adjacent to hydroxyl undergo markedly greater broadening than the lines assigned to both secondary methylene and methyl groups.

For 1-butanol and 2-butanol the effects of line broadening were especially strong as compared with the other alcohols. A BSA concentration of around 5% caused a broadening effect already leading to the fusion of the individual lines in multiplets. For 1-butanol and 2-butanol, therefore, the comparative line width analysis was performed in a BSA concentration of 0–4% instead of the 0–10% usually applied. Parts A–D of Figure 3, in which the results for four isomeric butanols are compared, gives a particularly clear illustration of the tendency toward selectivity in the NMR line width increase for different fragments of the alcohol molecules and also toward the effect of alcohol branching on the line broadening.

It is apparent that long-chained alcohols undergo the greatest broadening as compared with the branched ones and also that the methylenes vicinal to the hydroxyl groups undergo (Figures 2 and 3) selectively greater broadening than other groups of protons.

NMR Line Width Results for Glycols. The comparative results of the line width measurements for the hydrophobic parts of glycols are shown in parts A–C of Figure 4. It is clear

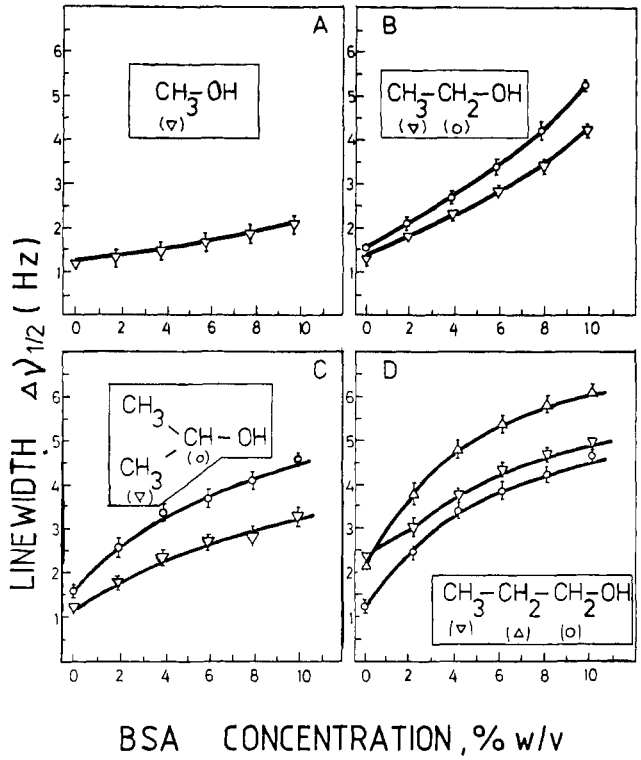

FIGURE 2: Line widths of NMR signals of the alcohols (3% v/v in D_2O) as a function of BSA concentration, pH 7.0. (A) Methanol, (B) ethanol, (C) 2-propanol, and (D) 1-propanol (pH 7.0). Each point represents the average of 25 measurements (five independently prepared samples, five NMR registrations each). The error indicated is the calculated standard deviation, which was omitted when it was lower than the point dimensions. The analyzed lines are as shown in Table I.

that in this case the increase in line widths with increased BSA concentration is more limited than that for monovalent alcohols. The lines of both ethylene and two propylene glycols (parts A–C of Figure 4) were found to be affected by BSA

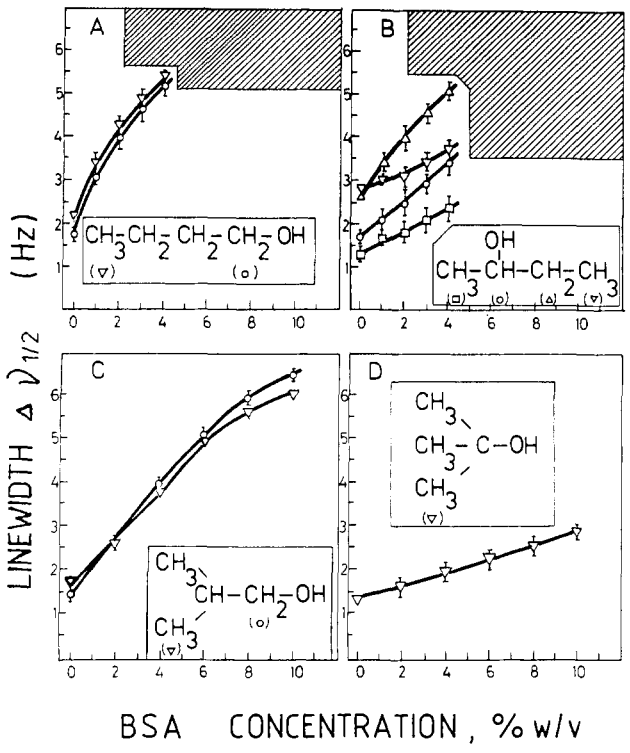

FIGURE 3: Line widths of NMR signals of isomeric butanols (3% v/v in D_2O) as a function of BSA concentration. (A) 1-Butanol, (B) 2-butanol, (C) 2-methylpropanol, and (D) 2-methyl-2-propanol (pH 7.0). Experimental averaging and error are as shown in Figure 2. The analyzed lines are as shown in Table I. Hatching shows the areas of greatest broadening where line widths are not measurable.

to a distinctly smaller extent than the monovalent alcohols of ethanol and propanol, having a corresponding hydrocarbon content (parts B–D of Figure 2). Moreover, in contrast to the effects observed for monovalent alcohols, the selectivity in line broadening for glycols is not marked. However, the general influence of the chain length on the line broadening is preserved when the propanediols (parts B and C of Figure 4) and ethanediol (Figure 4A) are compared.

Selectivity in Relative Line Broadening. The NMR experiments in the system alcohol–BSA–D_2O were carried out at a constant concentration of each alcohol (3% v/v) and at various BSA concentrations (0–10% or 0–4% w/v). The resulting ranges of alcohol–BSA, i.e., the number of molecules of the respective alcohols "attacking" one protein macromolecule, vary slightly for different alcohols (Table I), being in the range of 200–4500.

BSA polypeptide chain contains ~600 amino acid residues, and the loop regions of each subdomain of this protein contain three helical segments, ~30% of the cylindrical surface being hydrophobic (Brown, 1975). Under the conditions of our experiment (Table I), around 0.33–7.5 alcohol molecules (depending on BSA concentration and the kind of alcohol) fall to each amino acid side chain of protein.

If one assumes that the alcohol molecules interact mainly or most strongly with hydrophobic regions of the protein, in our experiment 1–21 alcohol molecules fall to each statistical hydrophobic side chain. This certainly fulfills in the situation when the protein macromolecule is not destabilized the optimal conditions for the contacts and binding between nonpolar portions of the alcohol molecule and the hydrophobic clusters of the protein side chains and for the exchange between free and bound alcohol molecules.

Therefore, the comparison of the relative line broadening as a function of the alcohol/BSA molar ratios is consistent with a physical situation promoting the events in protein macromolecule destabilization.

Thus, we recomputed the direct line width results from the experimental curves (parts A–D of Figures 2 and 3) for the relative line broadening in the applied ranges (Table I) of the molar ratios of alcohol/BSA.

The results are presented (Figure 5) separately for three groups of protons characterized by their location in the alcohol molecule as follows: (A) group of protons in methylenes or methines vicinal to the hydroxyls; (B) protons of methyl groups mostly separated from hydroxyls; and (C) protons of groups other than A or B.

It may be seen that relative broadening of the lines for various alcohols, being somewhat dependent on the alcohol/BSA molar ratio range, roughly follows the pattern

2-propanol ≤ 2-butanol < ethanol < 2-methylpropanol < 1-propanol < 1-butanol (1)

2-butanol < 1-propanol ≤ ethanol ≤ 2-propanol < 2-methylpropanol < 1-butanol (2)

2-methyl-2-propanol ≤ methanol < 2-butanol < 1-propanol (3)

for the proton groups A, B, and C, respectively.

Quantitatively, the relations shown in eq 1–3 together with comparative line broadening for glycols are illustrated in Figure 6, where the magnitudes of broadening for a 1000:1 alcohol/BSA molar ratio (i.e., when an average of 1 to 2 alcohol molecules fall to one amino acid residue in the BSA

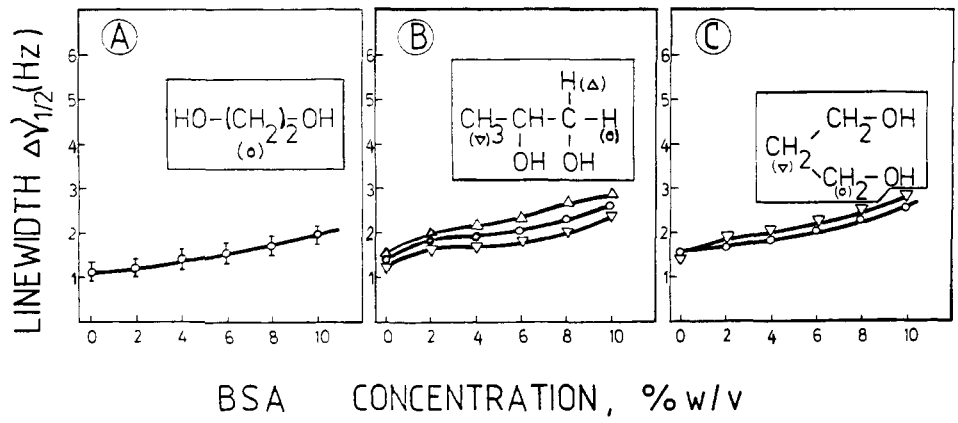

FIGURE 4: Line widths of NMR signals of glycols (3% v/v in D_2O) as a function of BSA concentration. (A) Ethanediol, (B) 1,2-propanediol, and (C) 1,3-propanediol (pH 7.0). Experimental averaging is as shown in Figure 2. The standard error in (B) and (C) (not included for graphic reasons) is not greater than that shown in (A).

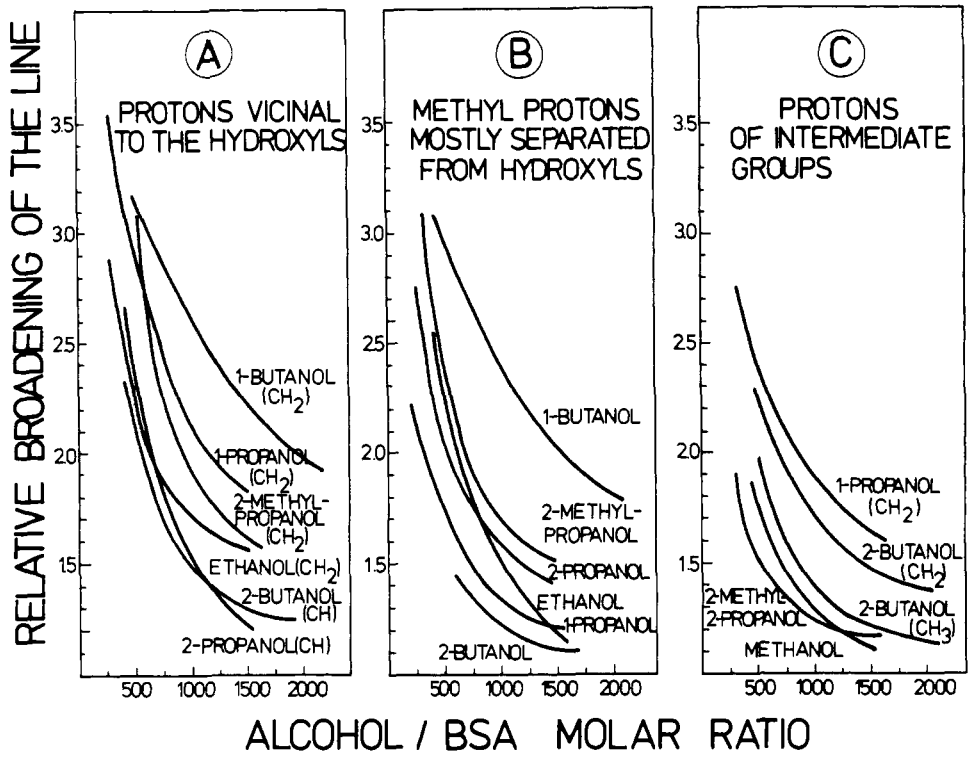

FIGURE 5: Dependence of the relative broadening of NMR signals for monovalent alcohols on the alcohol/BSA molar ratio.

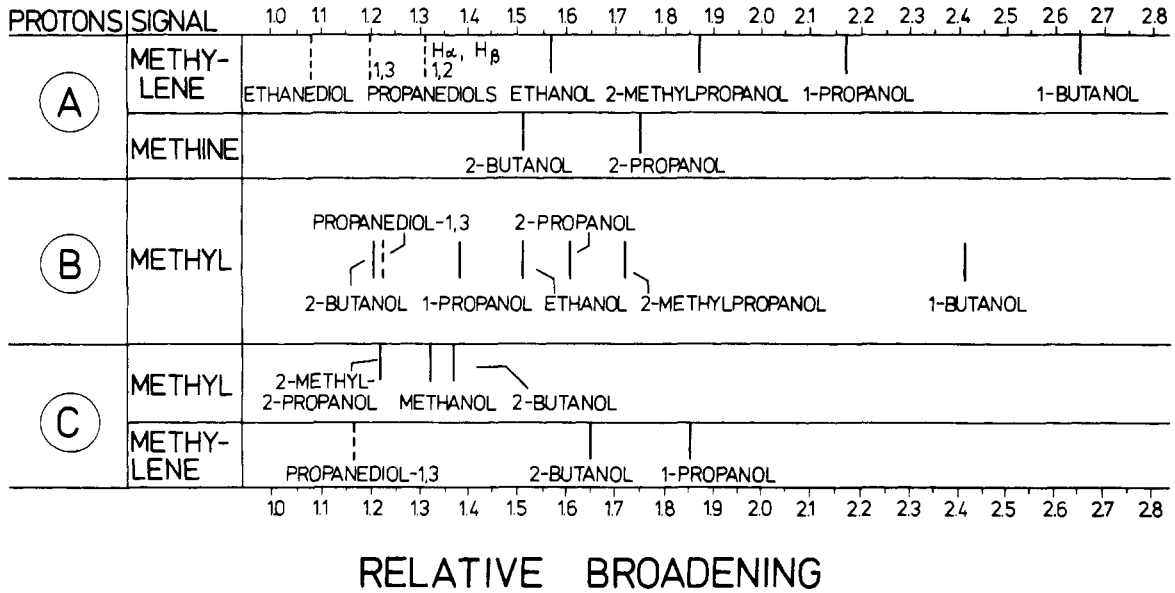

FIGURE 6: Quantitative indication of ranking in the association effectivity of various segments of alcohols with BSA, as judged from relative line broadening for a 1000:1 molar ratio: (solid bars) results for monovalent alcohols; (dotted bars) results for glycols.

chain) are presented. Figures 5 and 6 reflect the remarkable difference in immobilization of the various hydrophobic fragments of the alcohol molecules upon their interaction with BSA. The protons in fragments of alcohol molecules adjacent to the hydroxyls (group A), especially in methylenes but also in methines, undergo much greater line broadening than those of groups B or C.

In the case of methyls, the broadening is especially great for 1-butanol (Figure 5B) and the least for mainly branched 2-methyl-2-propanol and for methanol, having the shortest alkyl chain (Figure 5C).

Also, the effects of the chain length, location of the hydroxyl, and branching of the hydrocarbon chain on the line broadening may be judged unequivocally from Figure 6.

Furthermore, the markedly smaller broadening for the NMR lines of glycols as compared with those of monovalent alcohols is very apparent.

Discussion

NMR Methodology. The utility of high-resolution NMR as a method for investigating the binding of small molecular species to proteins is well recognized and well reviewed (Roberts & Jardetzky, 1969; Hayes et al., 1974).

The part of a small ligand molecule which is preferentially stabilized by interaction with a protein macromolecule can be readily identified because it is characterized, in comparison with the remainder of the molecule, by a longer correlation time and consequently by larger increments in the relaxation

rates and a much broadened spectral line (Jardetzky, 1964).

If the exchange of ligand, i.e., in our case alcohol molecules, between free (F) and bound (B) phases (F $\rightleftarrows$ B) is fast, the following relation (Zimmerman & Brittin, 1957; Jardetzky, 1964) holds:

$$\left(\frac{1}{T_i}\right)_F < \left(\frac{1}{T_i}\right)_B < \frac{1}{T} \qquad (4)$$

where T is the average lifetime in either F and B phase, $1/T$ is the rate of exchange F $\rightleftarrows$ B, and T_i is the corresponding relaxation time, longitudinal ($i = 1$) and transversal ($i = 2$), in the F and B phases, respectively.

The resulting relaxation rate, proportional to the spectral line width at half-height ($\Delta\nu_{1/2}$), is then a weighted average between the rates for F and B phases, according to the formula

$$\Delta\nu_{1/2} \sim \frac{1}{T_i} = \left(\frac{p}{T_i}\right)_B + \left(\frac{1-p}{T_i}\right)_F \qquad (5)$$

where p is the fraction of the ligand molecules in the bound phase.

In several systems examined by the method outlined above, the fast exchange between free and bound phases has most often been considered, especially for the weak complexes (Jardetzky & Wade-Jardetzky, 1965; Fisher & Jardetzky, 1965; Hollis, 1967; Gerig, 1968; Roberts & Jardetzky, 1969).

In our case, the absence of chemical shift changes at increased BSA concentration over the entire aliphatic range of the alcohol NMR spectra is in accord with the fact that alcohols do not upon interaction with protein macromolecules form any kind of strong complexes. However, considering the marked and selective broadening of the NMR lines of the alcoholic denaturants in the presence of BSA, we may suppose that they reflect the increase in the correlation time of the denaturant molecules, i.e., the existence of the weak hydrophobic associations between the alcohols and BSA fragments.

The assumption about sufficiently fast exchange between the bound and free alcohol molecules is easily fulfilled because nonspecific interaction of alcohols with proteins (St. Pierre & Jencks, 1969; Herskovits et al., 1970) can lead to the formation of very weak associates and the excess of alcohol (i.e., the free ligand phase) is essential in the model of experiments for solvent perturbation study.

It is evident that the observed broadening of the alcohol lines cannot be attributed to any unspecific mechanism such as, for example, the change in external viscosity of the protein solution because this would not explain the selectivity in the line width increase.

Although the line width of tested single lines of higher multiplets certainly reflects, similarly as for the singlets and doublets, the change in the relaxation rates of the individual fragments of the alcohols upon their interaction with the protein, in order to prove this it would be desirable to measure the segmental relaxation times T_1 or T_2 from proton and ^{13}C NMR.

NMR spectroscopic studies have occasionally been undertaken in systems where denaturing agents or alcohols served as probes for NMR experiments (Hollis, 1967; Fung & Sarney, 1971; Randall et al., 1972) but generally in approaches quite different from ours.

Hollis (1967) followed the effects of the enzyme yeast alcohol dehydrogenase (YADH) on the NMR spectra of ethanol. The use of ethanol was informative regarding the mechanism of substrate–enzyme binding and the effect of coenzymes.

In the binding experiments with membrane proteins of the erythrocytes (Randall et al., 1972) benzyl alcohol was chosen as the NMR probe, giving information concerning membrane fractions containing the binding sites.

Fung & Sarney (1971) investigated by ^{14}N NMR the interaction between BSA and the denaturants urea and guanidinium hydrochloride (Gdn·HCl), but at concentrations producing a large extent of protein denaturation (1–2 and 2–6 M for Gdn·HCl and urea, respectively). The significant (~30%) broadening of the line widths of ^{14}N resonances of both denaturants with increasing BSA concentration was attributed by these authors to the association between BSA and the denaturing agents.

The aim of our investigation was to demonstrate the feasibility of extending the application of NMR to the study of the specific interaction of proteins with typical hydrophobic perturbants leading to destabilization of the protein structure. In such a study the biochemically significant concentration of a solvent perturbant should in general be higher than in examination by NMR of the drug–receptor or substrate–enzyme complexes.

It is therefore clear that this kind of NMR study does not require a very high resonance frequency and, moreover, that the use of averaging NMR techniques is not essential for the interpretation of results.

Relationship between Denaturing Power and Association of Alcohols with Proteins. The overall broadening trend of the NMR lines of aliphatic alcohols in the presence of BSA supports the view concerning stronger immobilization by the protein of unbranched and longer chains of the alcohols. An interesting feature is that the effectiveness of association roughly follows the denaturation effectiveness of these alcohols for the globular proteins (Herskovits et al., 1970). Quantitatively, the relation of effectiveness for monovalent alcohols and glycols is illustrated in Table II. Both association and denaturing ability have been calculated with respect to 1-butanol, which was found in the analyzed group of alcohols to be the most effective denaturant (Herskovits et al., 1970) and also showed the strongest association with BSA (Figures 4A, 5, and 6).

Although different globular proteins and results from different techniques have been taken into consideration, the results (Table II) are surprisingly parallel—both the association and the denaturation properties are increased together with the length of the hydrocarbon chain of the alcohol and decreased for the branched molecules.

Thus, the evidence from NMR spectroscopy supports the hydrophobic mechanism of the alcohol action, which was suggested by many workers using different techniques. However, the differences in immobilization of the various parts of the alcohol molecules upon binding to the protein reflect also the nature of binding sites and the influence of the hydroxyl groups on hydrophobic association. These differences have not been demonstrated directly by other methods.

The pattern of quantitatively low line broadening for glycols would explain their reduced efficiency for association with a protein as compared with the weaker hydrogen-bonding solvents.

Thus, it is evident that though the location of the hydroxyl influences the hydrophobic alcohol–protein interaction, the greater ability for the formation of multiple hydrogen bonds by dihydroxy alcohols does not promote their association with apolar fragments of the BSA chain.

The poorer ability to associate with a protein is in general agreement with the low denaturation effectiveness of glycols

Table II: Comparison of the Relative[a] Effectiveness of Denaturing Power of Mono- and Dihydroxy Alcohols with Their Relative[a] Effectiveness for Hydrophobic Associations with BSA

no. of carbon atoms	alcohol	rel effectiveness of denaturing power of globular proteins[b] (%) for α-chymotrypsinogen	rel effectiveness of denaturing power of globular proteins[b] (%) for myoglobin	rel effectiveness of hydrophobic associations with BSA (%) group A, CH_2 or CH	group B, CH_3	group C, CH_3 or CH_2
1	methanol	9.7	6.5			17.9
2	ethanediol	6.4	5.6	22.2		
	ethanol	18.4	15.1	34.3	32.9	
3	1,3-propanediol	8.2	9.5	30.9	30.4	
	1,2-propanediol			35.8	35.3	
				35.4		
	2-propanol	26.9	23.5	56.7	52.9	
	1-propanol	43.8	40.0	68.4	40.5	53.9
4	2-methyl-2-propanol	35.0	33.0			48.1
	2-butanol	63.6		64.2	43.2	53.2 (CH_3)
						64.4 (CH_2)
	2-methylpropanol	87.5	100.0	81.3	65.6	
	1-butanol	100.0	100.0	100.0	92.5	

[a] With respect to 1-butanol. [b] Calculated from the data of Herskovits et al. (1970).

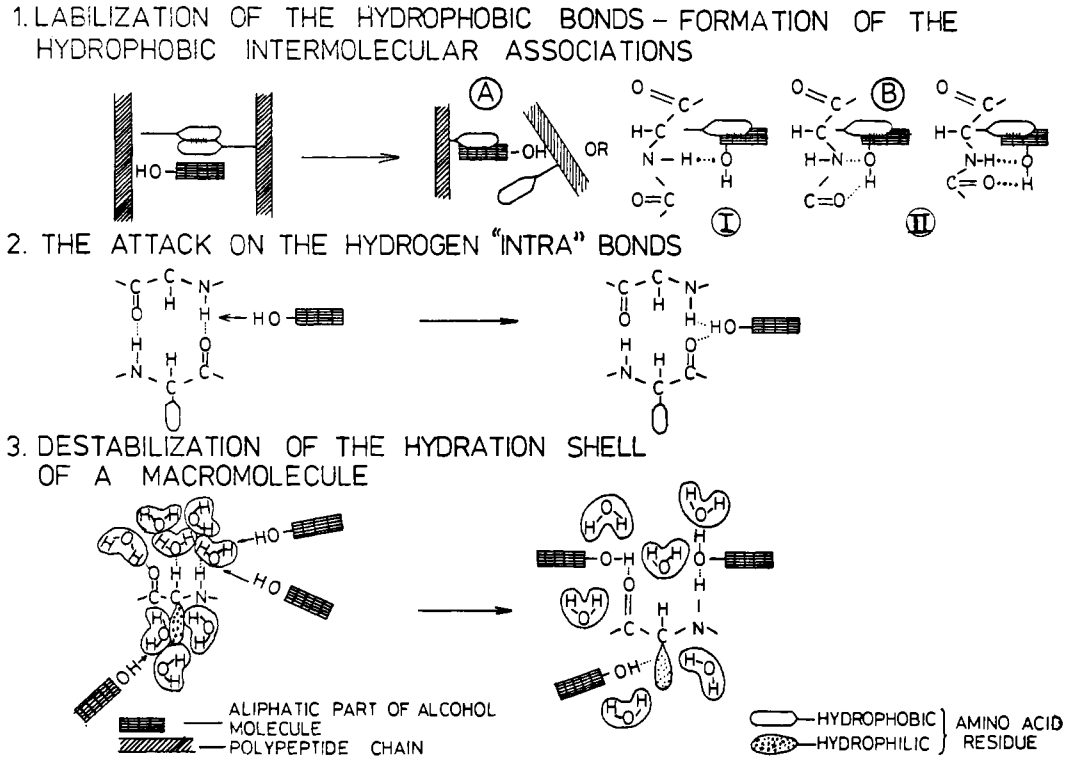

FIGURE 7: "A priori" mechanism of the destabilizing effect of aliphatic alcohols on the structure of globular proteins. (1A) Pure hydrophobic associations, without extra supporting bonds, and (1B) hydrophobic associations with additional stabilization by the hydrogen bonds between the hydroxyls and the peptide bonds [(I) single hydrogen bond; (II) two hydrogen bonds]; (2 and 3) formation of other structures with hydrogen bonds in which the alcohol hydroxyls participate.

for many proteins (Herskovits et al., 1970).

Interaction of Alcohols with BSA Structural Implications. It is possible to arrive at a realistic model for the association of alcohols and proteins consistent with the NMR broadening data presented here. Let us consider first some of the probable events resulting from the protein–alcohol interactions which lead eventually to the unfolding of the protein (Figure 7). On the basis of the line broadening data of the NMR spectra, we can estimate how important the various interactions might be.

The quantitative pattern of the line broadening for several nonpolar fragments of the alcohol molecules indicates that the greatest broadening was observed for protons of the methylenes vicinal to the hydroxyls. However, the latter effect would be expected not for pure hydrophobic associations (Figure 7, 1A) but rather for the intermolecular hydrophobic complexes with additional stabilization due to hydrogen bonding.

The extra stabilization appears to be due to the involvement of the hydroxyl groups in the hydrogen-bonding interactions with polar portions of the peptide bonds (Figure 7, 1B). In detail a choice between "pure" (type A) and "stabilized" (type B) hydrophobic association can be made as follows. For type A different apolar portions of alcohol molecule would be subjected to similar changes in the line broadening. Thus, the observed order in line broadening, $CH_3 > CH_2 > CH$, should be preserved. The B interactions would be expected to give rise to a stronger broadening effect for the segments of the chains which are vicinal to the hydroxyls because the additional hydrogen bonding can definitely immobilize these segments.

In fact, the latter effect would appear to be dominant for primary or unbranched alcohols. The evidence for such in-

teraction lies in the surprisingly larger broadening of the NMR lines of methylenes closest to the hydroxyls as compared with the rest of the nonpolar chain.

One can suppose that during the alcohol–protein interaction participation of hydroxyl groups in bonding leading to formation of the structures shown in parts 2 and 3 of Figure 7 might possibly also influence the line width of hydrophobic fragments vicinal to the hydroxyls. However, comparison of the results for mono- and dihydroxy alcohols tends to rule out such a possibility.

In contrast to monohydroxy alcohols, glycols can be involved in interaction with the protein by the hydrogen bonds forming interchain cross-links. Together with water dipoles they are capable of producing the extended hydrogen-bonded networks linked to the macromolecules [for a review, see Franks & Eagland, 1975)]. However, the strongly decreased ability to associate with BSA observed for glycols relative to alcohols suggests that hydrophobic association is not promoted by formation of the multiple hydrogen bonds.

Additional evidence and insight concerning the mode of association of alcohols with proteins are given by a comparison of the results obtained for the various isomers of propanols and butanols. For example, 2-methylpropanol appears to be less effectively associated with BSA than 1-butanol or the shorter but unbranched 1-propanol. Reduced ability for association is also noted for the secondary alcohols 2-propanol and 2-butanol, indicating that the location of the hydroxyl group with respect to the chain is a primary factor favoring or reducing the binding of an alcohol to protein. Here the aliphatic chain branching seems to be of secondary importance.

Significantly, the coincidence of the chain branching and central location of the hydroxyl in tertiary butanol (2-methyl-2-propanol) seems to be the cause of the strongest reduction in its association with the protein.

The above results can be attributed to steric effects. The branched alcohol molecule is hindered in its approach to the nonpolar side groups of the protein in a way that is insufficient for the occurrence of multiple hydrophobic bonding.

It seems that the latter effect is responsible for lower immobilization of the respective methine groups in secondary alcohols as compared with that of methylenes in primary alcohols. In this situation for different fragments of the alcohol chain a more symmetrical binding to BSA may be suggested.

Subsequently, though in 2-propanol the binding of the CH group still appears stronger than that of the two methyl groups, 2-butanol adopts on binding the conformation favoring a strong attachment to BSA, not only for the CH group but also for its nearest neighbors located on both sides of the hydroxyl group (CH_2 and CH_3, respectively). This reflects also the additional effects of the hydroxyl group on the protein–alcohol interaction.

Several years ago Schrier et al. (1965) suggested that binding of nonpolar groups of an alcohol may be to clusters consisting of several nonpolar amino acid residues which can anchor an alcohol by multiple hydrophobic bonds. For example, two leucyl residues can form a slot with multiple hydrophobic bonds for the butyl group, etc.

In light of our results and of the three-dimensional structure of BSA based on sequence analysis and model binding studies (Brown, 1975), the suggestions mentioned above are quite applicable for association between BSA and alcohols. In the domain structure of BSA (Brown, 1975), three peptide segments are arranged parallel to each other, forming a long hydrophobic groove. This groove forms an especially favorable orientation for the accommodation of several alcohol molecules with multiple hydrophobic bonds.

A type of stabilized hydrophobic association could result from the additional involvement of a single hydrogen bond between the alcohol hydroxyl and NH or C=O group and also from the formation of two hydrogen bonds (Figure 7, B). The latter effect would require a change in the common transplanar conformation of the peptide bond, which is considered to be relatively unlikely. The carbonyl oxygen which constitutes part of the exposed surface of the polypeptide backbone (Lee & Richards, 1971; Shrake & Rupley, 1973) in native proteins is the most likely acceptor of the OH group.

In the domain structure of BSA (Brown, 1975) the outer helical surfaces of the hydrophobic grooves are highly polar and at their ends clusters of positively charged amino acid sides are situated. The latter residues could potentially participate in formation of the hydrogen bonds with the oxygen atom of the alcohol hydroxyl groups. However, the above hydrogen bonds are probably not of primary importance in extra stabilization of the hydrophobic interaction. Being more probable for glycols, they do not influence sufficiently the association of glycols with protein. In fact, the hydroxyl groups seem to prevent the penetration of glycol molecules into the hydrophobic interior of a protein. This is in general agreement with the data of Nozaki & Tanford (1965), who demonstrated low solubilization of hydrophobic side chains of proteins for glycols. Interaction of hydrocarbons with apolar residues of globular proteins was followed by experimental techniques based on the hydrocarbon solubility (Wishnia, 1969a,b; Robillard & Wishnia, 1972a) and on ^{19}F NMR for halogenated hydrocarbon (Robillard & Wishnia, 1972b). However, although attempts to compare the data for hydrocarbon binding with those on alcohol denaturation have occasionally been undertaken, they did not give a predictable correlation [for a review, see Franks & Eagland (1975)]. Hydrocarbons are nearly insoluble in water, hence their ineffectiveness as denaturants.

Our results strongly confirm the hydrophobic mechanism of the interaction of alcohols with proteins. However, while indicating redistribution of the strength of this interaction along the chains and molecule branches, our results also demonstrate the influence of the vicinity of the hydroxyl group toward the promotion of the hydrophobic association. For a more general acceptance of the suggested model, much remains to be learned from the similar studies for other globular proteins. Nevertheless, one can conclude that NMR spectroscopy does provide a fair degree of insight into the nature of the protein–alcohol interaction.

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Structural Comparisons of Heme Binding Proteins†

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ABSTRACT: Of the 82 three dimensionally characterized residues of cytochrome c_{551}, 49 are found to be structurally and topologically equivalent to the globin fold and 41 are equivalent to the cytochrome b_5 fold, with a respective root mean square separation of 3.5 and 4.9 Å between equivalenced C_α atoms. The common fold represents a central heme binding core, corresponding to the middle exon of certain globin genes. After superposition of the protein folds, the heme irons are found to be separated by 5.4 and 1.6 Å, while their heme normals are inclined by 6° and 32°, respectively. Furthermore, the heme "face", determined by the asymmetric attachment of the vinyl and propionyl side chains, is directed similarly in all three heme proteins. The heme itself is rotated by 72° and 116° about its normal, respectively. The minimum base change per codon for the three pairwise comparisons corresponds to the expected value of random sequence comparisons. While all three heme proteins may have diverged from a common ancestor, their similarity may have arisen from the requirements of heme binding or the utilization of a particularly stable fold. Known structures within commonly accepted divergent families were superimposed in order to discriminate better between convergence and divergence. Minimum base changes per codon, number of deletions and insertions, percentage of equivalenced residues, precision of heme superposition, and root mean square separation of equivalenced C_α atoms were tested as measures of evolutionary relationships.

The three-dimensional structures within three families of heme binding proteins have been well studied: the globins, the cytochrome *c* family, and the mammalian cytochrome b_5 from the endoplasmic reticulum. Rossmann & Argos (1975) have shown a reasonable structural similarity between the globin fold and cytochrome b_5. This paper demonstrates that a comparable structural similarity exists between the cytochrome *c* and globin folds.

The globin family extends further than the oxygen carriers hemoglobin and myoglobin found in higher vertebrates. Proteins with the same function and homologous structure have been found in the annelid worm *Glycera dibranchiata* (Padlan

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