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Ligand–Apomyoglobin Interactions

CONFIGURATIONAL ADAPTABILITY OF THE HAEM-BINDING SITE

By KIRSTEN E. LIND and JESPER V. MØLLER
Institute of Medical Biochemistry, University of Aarhus, 8000 Aarhus C, Denmark

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1. The interaction of the haem-binding region of apomyoglobin with different ligands was examined by ultrafiltration, equilibrium dialysis and spectrophotometry, to study unspecific features of protein–ligand interactions such as they occur in, for example, serum albumin binding. 2. Apomyoglobin, in contrast with metmyoglobin, binds at pH 7, with a high affinity, one molecule of Bromophenol Blue, bilirubin and protoporphyrin IX, two molecules of *n*-dodecanoate and *n*-decyl sulphate and four molecules of *n*-dodecyl sulphate and *n*-tetradecyl sulphate. 3. The number of high-affinity sites and/or association constants for the alkyl sulphates are enhanced by an increase of hydrocarbon length, indicating hydrophobic interactions with the protein. 4. Measurements of the temperature-dependence of the association constants of the high-affinity sites imply that the binding processes are largely entropy-driven. 5. Binding studies in the presence of two ligands show that bilirubin plus Bromophenol Blue and dodecanoate plus Bromophenol Blue can be simultaneously bound by apomyoglobin, but with decreased affinities. By contrast, the apomyoglobin–protoporphyrin IX complex does not react with Bromophenol Blue. 6. Optical-rotatory-dispersion measurements show that the laevorotation of apomyoglobin is increased towards that of metmyoglobin in the presence of haemin and protoporphyrin IX. Small changes in the optical-rotatory-dispersion spectrum of apomyoglobin are observed in the presence of the other ligands. 7. It is concluded that the binding sites on apomyoglobin probably do not pre-exist, but appear to be moulded from predominantly non-polar amino acid residues by reaction with hydrophobic ligands. 8. Comparison with data in the literature indicates that apomyoglobin on a weight basis has a larger hydrophobic area available for binding of ligands than has human serum albumin. On the other hand, the association constants of serum for the ligands used in this study are generally somewhat larger than those of apomyoglobin.

Despite extensive investigations into the interaction of serum albumin with different ligands, the detailed nature of the high-affinity binding sites of the protein has not been elucidated. Although the binding sites of this protein are characterized by variations in the binding affinity for various ligands, the difference in chemical structure of compounds that are bound by common binding sites, as evidenced from competition experiments, is impressive (Kragh-Hansen *et al.*, 1974; Cunningham *et al.*, 1975). The formation of such complexes contrasts with that of most other proteins, for example enzymes, which most often exhibit a high degree of binding specificity towards their substrates. The predominating view is that the unspecific nature of interaction of serum albumin with different ligands is to a large extent caused by interaction with 'patches' or 'clefts' of non-polar amino acid residues on the surface of the protein, and that the stability of the complexes is derived from hydrophobic interactions [see, for instance, Tanford (1972)].

An interesting example of a protein that exhibits features of both specific and unspecific binding is provided by myoglobin. X-ray-crystallographic studies have shown that the haem group of myoglobin is situated in a 'pocket' of the native structure, which is lined mainly by non-polar amino acid residues (Kendrew, 1962). The haemin is firmly attached to metmyoglobin [the association constant is of the order 1×10^{12}–1×10^{15} M^{-1} (Bannerjee, 1962)], but the prosthetic group may be released by a reversible denaturation of the protein after exposure to an acid pH (Theorell & Ehrenberg, 1951; Shen & Hermans, 1972). After removal of the haemin by extraction with organic solvent and dialysis the resulting apomyoglobin is capable of binding with a relatively high affinity an equivalent amount of different dyes (Stryer, 1965; Ka Luk, 1971) by a process that resembles the interaction with serum albumin. For instance 1 mol of 8-anilino-1-naphthalene sulphonate is bound by 1 mol of apomyoglobin (17200 g) with an association constant of

290000 M^{-1} (Stryer, 1965), and 5 mol of the fluorescent dye is bound by 1 mol of serum albumin (67000 g) with an association constant of about 100000 M^{-1} (Daniel & Weber, 1966; Kragh-Hansen *et al.*, 1974). The hydrodynamic properties (Breslow, 1964) and rotational relaxation time (Stryer, 1965) of apomyoglobin indicate no gross conformational differences from metmyoglobin. Optical rotatory-dispersion and circular-dichroism measurements indicate a somewhat lower helix content of apomyoglobin than of metmyoglobin (Harrison & Blout, 1965; Breslow *et al.*, 1965; Atassi & Singhal, 1972), but the stoicheiometric dye-binding properties of the apoprotein makes it reasonable to assume that the hydrophobic area that interacts with haemin in the native structure is largely maintained in the absence of haemin. Further, it has been found that apomyoglobin combines with haemin to form metmyoglobin, with spectral properties similar to those of the native protein (Breslow, 1964; Harrison & Blout, 1965).

The properties of the apomyoglobin molecule mentioned above indicate that it may probably serve as a model substance with which to study specific and unspecific aspects of binding of ligands by proteins. In the present work the details of the interaction of apomyoglobin with ligands as diverse as Bromophenol Blue, aliphatic sulphates, dodecanoate, bilirubin and protoporphyrin IX were studied, and the results obtained were compared with the binding of these ligands by serum albumin.

Materials and Methods

Sperm-whale metmyoglobin was obtained from Miles Laboratories, Cape Town, South Africa, and aqueous solutions of the protein were deionized by passage through a mixed bed resin (AG 501 X8; Bio-Rad Laboratories, Richmond, CA, U.S.A.). Removal of haemin from metmyoglobin was done by extraction with methyl ethyl ketone in acid solution as described by Teale (1959). The aqueous phase of the extracted solution was dialysed against water overnight, after which the solution was deionized and freeze-dried. The freeze-dried preparation was stored at 4°C until used.

The compounds to be tested for binding by apomyoglobin and myoglobin were obtained from the following sources: Bromophenol Blue (Merck AG, Darmstadt, W. Germany); haemin and bilirubin (Sigma Chemical Co., St Louis, MO, U.S.A.); protoporphyrin IX (Calbiochem, San Diego, CA, U.S.A.); dodecanoic acid (Fluka AG, Buchs, Switzerland). The reagents were analytical grade and were used without further purification. Sodium *n*-dodecyl sulphate (95%) was from Sigma. The reagent was re-crystallized from ethanol after which it appeared homogeneous by t.l.c. (Mangold & Kammereck, 1962). The detergents *n*-decyl sulphate and *n*-tetradecyl sulphate were obtained from Cambrian Chemicals Ltd., Croydon, Surrey, U.K. Dodecyl [^{35}S]sulphate and [1-^{14}C]dodecanoate were obtained from The Radiochemical Centre, Amersham, Bucks., U.K. In some experiments, protoporphyrin IX, prepared from the dimethyl ester (Sigma) as described by Mauk & Girotti (1973), was used.

Binding experiments

The freeze-dried apomyoglobin preparation and metmyoglobin were in most cases dissolved in 0.033 M-sodium phosphate buffer, pH 7.0. The solutions were used for up to 2 days after preparation. Undissolved apomyoglobin and apomyoglobin that precipitated on standing were removed by centrifugation. Soluble apomyoglobin by gel chromatography on Sephadex G-200 (Lind *et al.*, 1974) was found to have a Stoke's radius of 2 nm (20 Å) indicating a monomeric species (Breslow, 1964). Binding of Bromophenol Blue by apomyoglobin and metmyoglobin was determined by ultrafiltration in a temperature-controlled centrifuge, as previously described (Kragh-Hansen *et al.*, 1972). The concentration of dye ranged from 0.02 to 0.5 mM, and that of the proteins from 0.02 to 0.03 mM.

Binding of dodecanoate and aliphatic sulphates was determined by equilibrium dialysis. A sample of protein solution (5 ml of 0.025–0.06 mM-apomyoglobin or metmyoglobin in 0.033 M-phosphate buffer, pH 7.0) was enclosed in a cellophane bag (Visking dialysis tubing, circumference 5 cm, boiled for 15 min in deionized water before use), and submerged in 5 ml of 0.033 M-phosphate buffer, pH 7.0, containing the compound to be tested for binding. The samples were shaken in a thermostatically controlled water bath for at least 24 h, which was shown in control experiments without protein in the bag contents to be a sufficient time-period for obtaining equilibrium conditions.

The binding of protoporphyrin was assessed by measuring the increase in light absorption at 403 nm of a 0.003–0.008 mM concentration of the compound in 0.033 M-phosphate buffer, pH 7.0, after addition of various amounts of the apomyoglobin solution to give molar ratios ranging from 0.1 to 4.0 of protein to protoporphyrin IX. After mixing, the samples were left for 1 h in the dark before the E_{403} was measured. The bound fraction at lower protein/protoporphyrin ratios, α, was calculated from the following equation:

$$\alpha = \frac{E_{obs.} - E_0}{E_\infty - E_0} \quad (1)$$

where $E_{obs.}$ is the observed absorption at a given protein concentration, E_0 is the absorption in the

absence of protein and E_∞ is the limiting value of absorption at high protein concentrations, corresponding to 100% binding of protoporphyrin IX.

The interaction of bilirubin with apomyoglobin was also measured spectrophotometrically on the basis of the increase in light absorption at 485nm. The concentration of bilirubin was 0.009mM, and the molar ratio of apomyoglobin to bilirubin ranged from 0.1 to 3.0. To avoid colloid formation of bilirubin (Brodersen & Theilgaard, 1969) the binding experiments were performed at pH9.0 rather than at pH7.0.

Analytical methods

The concentration of the following substances was determined on the basis of their molar absorbances: protoporphyrin IX, 263000 litre·mol^{-1}·cm^{-1} at 409nm in 2.7M-HCl (Falk, 1964); bilirubin, 52000 litre·mol^{-1}·cm^{-1} at 438nm in 0.1M-sodium phosphate at pH9.0 (Chen, 1973); metmyoglobin, 34500 litre·mol^{-1}·cm^{-1}; apomyoglobin, 15900 litre·mol^{-1}·cm^{-1} at 280nm in a pH6.8 phosphate buffer (Harrison & Blout, 1965); Bromophenol Blue, 78000 litre·mol^{-1}·cm^{-1} at 590nm in a 0.033M-phosphate buffer, pH7.0.

Radioactivity of dodecyl[^{35}S]sulphate and [1-^{14}C]-dodecanoate was determined by liquid-scintillation counting in a Packard Tri-Carb counter (model 3314). A small volume of sample (usually 0.5ml) was added to 10ml of the scintillator described by Bray (1960), and the samples were counted until the statistical uncertainty was less than 1%. The efficiency of counting was determined by addition of internal standard. Chemical analyses of aliphatic sulphates were performed by the Methylene Blue/chloroform extraction method (Ray *et al.*, 1966).

Optical-rotatory-dispersion measurements

Optical-rotatory-dispersion curves of apomyoglobin and myoglobin solutions were recorded on a FICA Spectropolarimeter instrument. The solution contained approx. 0.18mg of protein/ml, dissolved in 0.033M-phosphate buffer (pH7.0). The reduced mean residue rotation, $[m']$, was calculated from the equation

$$[m'] = \frac{3}{n^2+2} \cdot \frac{MRW}{lc} \cdot \alpha_\lambda \qquad (2)$$

where n is the refractive index (1.388 at 233nm), l is the light-path length measured in dm, c is the concentration of protein (g/100ml), α_λ is the observed rotation at wavelength λ, MRW is the mean molecular weight of the amino acid residues (112 and 115 for apomyoglobin and myoglobin respectively).

Results

Binding of Bromophenol Blue by apomyoglobin and metmyoglobin

Binding curves of Bromophenol Blue by apomyoglobin and metmyoglobin at pH7.0 and 25°C are shown in Fig. 1(*a*). The dye interacts only weakly with myoglobin, and the average number of molecules of Bromophenol Blue bound per molecule of protein ($\bar{\nu}_{BPB}$) is proportional to the concentration of free dye, [BPB_f], in the range of concentrations examined (0–0.2mM-Bromophenol Blue). By contrast, large amounts of Bromophenol Blue are bound by apomyoglobin at concentrations of uncombined dye below 0.02mM, but at higher concentrations, the binding curves of apomyoglobin and myoglobin have a

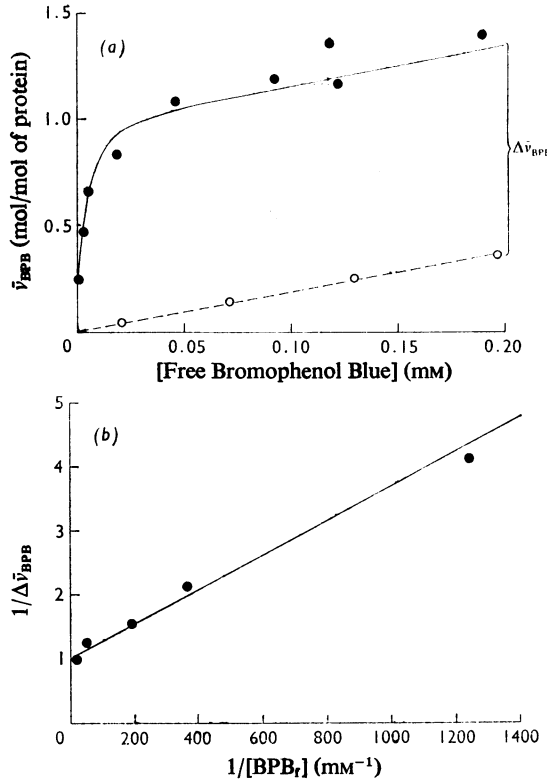

Fig. 1. *Binding of Bromophenol Blue by apomyoglobin* (●) *and metmyoglobin* (○) (*a*) *and Klotz plot of the high affinity binding of Bromophenol Blue by apomyoglobin* (*b*)

The experiments were carried out in 0.033M-phosphate buffer (pH7.0) at 25°C. The solid curve is calculated on the basis of one high-affinity site on apomyoglobin with an association constant of 3.7×10^5M^{-1} and weak affinity binding of Bromophenol Blue to the same extent as by metmyoglobin. Each point denotes the average of duplicate determinations from three experiments.

parallel course. It may therefore be assumed that weak binding of Bromophenol Blue by both forms of the protein occurs to the same extent. By subtracting $\bar{\nu}_{BPB}$ of myoglobin from that of apomyoglobin it should therefore be possible to estimate the interaction of the dye with the haem-binding site of apomyoglobin. The results of such an analysis are shown in the Klotz (1946) plot of Fig. 1(*b*). Apomyoglobin has one high-affinity binding site for Bromophenol Blue. The association constant is calculated from the slope of the line to be $3.7 \times 10^5 M^{-1}$ under the conditions of the experiment.

The effect of pH on binding of Bromophenol Blue by apomyoglobin and myoglobin is shown in Fig. 2. An increase in pH from 7 to 9 is only accompanied by a small decrease in the binding of the dye by both forms of the protein. However, at pH6.5, $\bar{\nu}_{BPB}$ of apomyoglobin is appreciably increased, indicating the presence of more than one strong binding site under these conditions. A further change of pH to 6.0 results in a pronounced increase in $\bar{\nu}_{BPB}$ at 0.02–0.06mM, but it should be noted that binding is decreased at concentrations of uncombined dye below 0.01mM. Drastic increases in dye binding at pH5.3 are observed for both apomyoglobin and myoglobin, and in these cases a precipitate of protein–dye complex is observed on standing. It can be concluded from these findings that in acid media the stoicheiometric interaction of Bromophenol Blue with apomyoglobin at pH7.0 is replaced by binding at several sites with a lower affinity for dye binding. As a consequence of these findings, acid media were avoided in all subsequent binding experiments.

Binding characteristics of different ligands

The analysis of binding curves of different ligands proceeded in a similar way as described for Bromophenol Blue above, and a summary of the results obtained is presented in Table 1. In contrast with Bromophenol Blue, aliphatic compounds have more than one strong binding site at pH7. In a homologous series of *n*-aliphatic alkyl sulphates, the addition of two methylene groups results in an appreciable increase in either the number of high-affinity sites (four strong sites for dodecyl sulphate compared with two for decyl sulphate) or in the magnitude of the association constants (compare tetradecyl and dodecyl sulphate). The enhanced reaction of long-chain alkyl sulphates with apomyoglobin points to an important role of hydrophobic interactions in the binding process. Dodecanoate in comparison with dodecyl sulphate interacts more

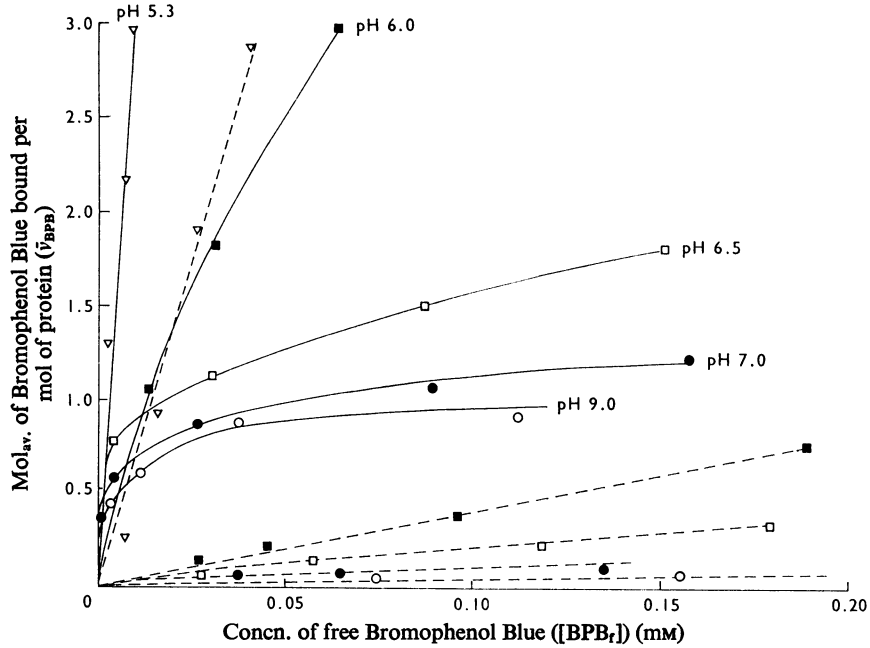

Fig. 2. *Effect of pH on binding of Bromophenol Blue by apomyoglobin* (——) *and metmyoglobin* (----)

The experiments were carried out in 0.033M-phosphate at 25°C. The symbols refer to the following pH values: ▽, 5.3; ■, 6.0; □, 6.5; ●, 7.0; ○, 9.0. Each point denotes the average of duplicate determinations from three experiments.

Table 1. *Number of high-affinity binding sites and association constants of diverse substances bound by apomyoglobin*

n and *K* denote the number of high-affinity binding sites and association constants respectively. Measurements were made in 0.033 M-phosphate at 25°C. The pH of the solutions was 7.0, except where otherwise indicated.

Ligand	*n*	$10^{-3} \times K$ (M^{-1})
Decyl sulphate	2	90
Dodecyl sulphate	2	100
	2	12
Tetradecyl sulphate	4	710
Dodecanoate	2	50
Bromophenol Blue	1	370
Bromophenol Blue (pH9.0)	1	130
Bilirubin (pH9.0)	1	900
Protoporphyrin IX	1	5500

weakly with apomyoglobin, having only two high-affinity binding sites with a somewhat smaller association constant than the strongest binding site of dodecyl sulphate. Among the other ligands presented in Table 1, only one high-affinity site is observed, and the association constant increases in the order Bromophenol Blue < bilirubin < protoporphyrin IX. No evidence was obtained for low-affinity binding of dodecanoate, bilirubin and protoporphyrin IX. By contrast, low-affinity binding of alkyl sulphates was discernible, as evidenced by non-saturatibility of the binding curves of apomyoglobin at high ligand concentrations and combination of the ligands with metmyoglobin.

It was decided to estimate enthalpy and entropy changes accompanying the binding, to evaluate further the role of hydrophobic forces in the binding process. Association constants for high-affinity binding of most of the ligands were measured at four different temperatures in the temperature interval 0–25°C. Logarithmic plots of the association constants as a function of the reciprocal of the absolute temperature ($1/T$) were found to be linear, enabling an estimate of the partial molal enthalpy change of the reaction, ΔH, from the van't Hoff equation

$$\ln \frac{K_1}{K_2} = \frac{\Delta H}{R}\left(\frac{1}{T_2} - \frac{1}{T_1}\right) \qquad (3)$$

where K_1 and K_2 refer to the association constants at temperatures T_1 and T_2. The entropy changes were calculated from:

$$\Delta S = (\Delta H - \Delta G)/T \qquad (4)$$

In this equation the standard free energy of the binding reaction, ΔG, is expressed in unitary units, i.e. by $RT \ln K'$, where K' is calculated in terms of mol fractions of the reactants in the solution ($K' = 55.6K$). The advantage of using the mol-fraction scale rather than molar concentration units for calculation of the thermodynamic parameters of association reactions in aqueous solutions has been discussed by Kauzmann (1959).

Table 2. *Thermodynamic parameters of the high-affinity binding of ligands by apomyoglobin*

The calculations are based on measurements of association constants in the temperature interval 0–25°C as described in the text. Note that ΔG is calculated in unitary units (see the text).

Ligand	ΔH (kJ/mol)	ΔG (kJ/mol)	ΔS ($J \cdot K^{-1} \cdot mol^{-1}$)
Dodecyl sulphate	−9	−39	101
Dodecanoate	−13	−37	81
Bromophenol Blue	0	−42	141
Bilirubin	−14	−44	101
Protoporphyrin IX	−12	−48	121

The results of the thermodynamic analysis are shown in Table 2. In all cases, the changes in enthalpy make a small contribution to the binding of the ligands, therefore the binding processes must be largely entropy-driven. According to Kauzmann (1959) these features are those expected for binding of ligands by primarily hydrophobic processes.

Inhibition experiments

Inhibitory relationships among bound ligands were characterized by determining the high-affinity binding curve of ligand A in the presence of a constant concentration of another ligand B. Assuming strict competitive relationships at a high-affinity site, the following equations apply:

$$\bar{\nu}_A(1) = \frac{K_A[A_f]}{1 + K_A[A_f] + K_B[B_f]} \qquad (5a)$$

$$\bar{\nu}_B(1) = \frac{K_B[B_f]}{1 + K_B[B_f] + K_A[A_f]} \qquad (5b)$$

where $\bar{\nu}_A(1)$ and $\bar{\nu}_B(1)$ are the average number of molecules of A and B bound by one molecule of apomyoglobin on this site; $[A_f]$ and $[B_f]$ are the concentrations of uncombined ligand, and K_A and K_B, which were taken from Table 1, are the association constants for binding on this site. Knowing the total concentration of B (uncombined plus combined ligand) and the concentration of apomyoglobin it is possible from eqns. (*5a*) and (*5b*) to calculate $\bar{\nu}_A(1)$ as a function of $[A_f]$. To obviate complications arising from the presence of several binding sites on apomyoglobin the ligands chosen for the experiments has only one or two high-affinity sites, and low-affinity binding was either weak or non-existent. The results are shown in Figs. 3–5,

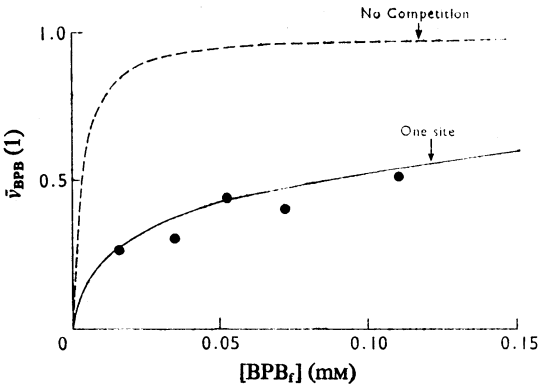

Fig. 3. *Effect of protoporphyrin IX on high-affinity binding of Bromophenol Blue by apomyoglobin*

The experiment was carried out in 0.033 M-phosphate buffer (pH 7.0) and 25°C. Experimental data (●) refer to binding of Bromophenol Blue in a medium containing 0.0150 mM-apomyoglobin and 0.0125 mM-protoporphyrin IX. ——, Calculated on the assumption of full competition between binding of Bromophenol Blue and protoporphyrin IX at the high-affinity binding site of apomyoglobin by the use of eqn. 5 and the association constants given in Table 1; – – – –, represents binding of Bromophenol Blue by apomyoglobin in absence of protoporphyrin, calculated from the association constant of the dye given in Table 1. The graph demonstrates representative results from one of four experiments.

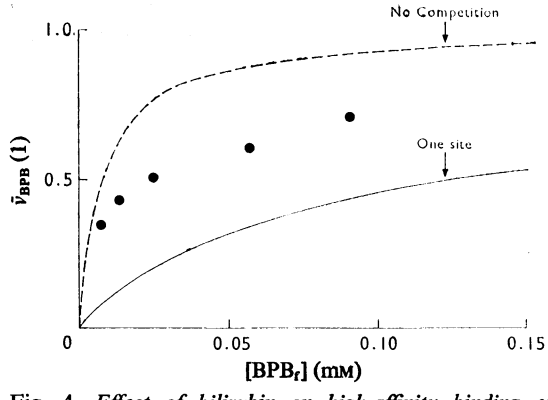

Fig. 4. *Effect of bilirubin on high-affinity binding of Bromophenol Blue by apomyoglobin*

The experiments were carried out in 0.033 M-phosphate (pH 9.0) and 25°C. Experimental data (●) refer to binding of Bromophenol Blue in a medium containing 0.0159 mM-apomyoglobin and 0.025 mM-bilirubin. ——, Calculated on the assumption of full competition between binding of Bromophenol Blue and bilirubin at the high-affinity binding site of apomyoglobin; the dashed curve represents high-affinity binding of Bromophenol Blue by apomyoglobin at pH 9.0 in the absence of bilirubin and is calculated on the basis of the association constants given in Table 1. Each point denotes the average of duplicate determinations from three experiments; – – – –, no competition.

in which binding curves calculated as described above have been compared with experimentally determined values. Fig. 3 shows that good agreement between the theoretical curve and experimentally determined values for binding of Bromophenol Blue (corrected for weak affinity binding) is obtained in the presence of protoporphyrin IX. On the other hand, bilirubin is less efficient in displacing the dye from its high-affinity binding site than expected on the basis of competitive inhibition (Fig. 4). In the presence of Bromophenol Blue, dodecanoate binding is inhibited to an extent corresponding approximately to one binding site (Fig. 5). However, the shallow shape of the binding curve, which can be drawn through the experimental points, suggests that the two binding sites on apomyoglobin exist in the presence of Bromophenol Blue, but with a decreased affinity for the fatty acid. As noted in the legend to Fig. 5, the concentration of free Bromophenol Blue was found to be practically constant at different concentrations of dodecanoate. It is therefore possible in this case to calculate apparent association constants and number of binding sites by a straightforward Klotz analysis of the data. Such a procedure verifies the existence of two binding sites for dodecanoate, and they have an apparent association constant of

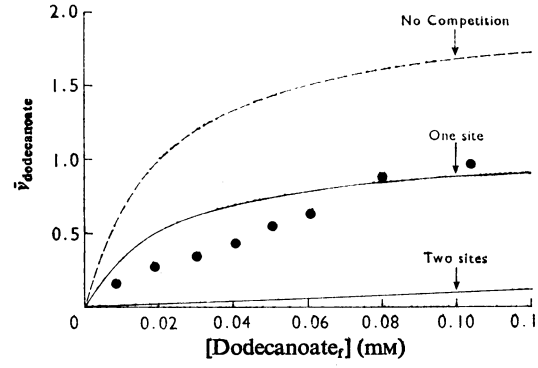

Fig. 5. *Effect of Bromophenol Blue on binding of dodecanoate by apomyoglobin*

The experiments were carried out in 0.033 M-phosphate buffer (pH 7.0) and 25°C. Experimental data (●) refer to binding of dodecanoate in a medium containing 0.0388 mM-apomyoglobin and 0.269 mM-Bromophenol Blue. The concentration of free Bromophenol Blue was found to be 0.241 mM (range 0.240–0.243 mM), independent of the concentration of dodecanoate. ——, Calculated on the assumption of full competition between binding of Bromophenol Blue and dodecanoate at one or two binding sites for dodecanoate on apomyoglobin, on the basis of the association constants given in Table 1. Each point is the average of duplicate determinations from two experiments.

Table 3. *Reduced mean residue rotation of metmyoglobin, apomyoglobin and complexes of apomyoglobin with different ligands*

Measurements were made using 0.01 mM protein solution in 0.033 M-phosphate buffer (pH 7.0), except for bilirubin, which was measured in 0.033 M-phosphate (pH 9.0).

Protein	Ligand	Ligand concentration (mM)	$\bar{\nu}$	$[m']_{233}$ without ligand	$[m']_{233}$ in presence of ligand	$\Delta[m']_{233}$ (%)	No. of observations
Metmyoglobin	None	—	—	9310±410	—	—	9
Apomyoglobin	Haemin	0.012	1.0	7170±410	8460±490	+18.0	5
	Protoporphyrin IX	0.014	1.0	7020± 50	7710±140	+ 9.8	4
	Bilirubin	0.030	1.0	7170±250	7520±370	+ 4.9	4
	Bromophenol Blue	0.060	1.1	7120±190	7270±340	+ 2.1	8
	Dodecanoate	0.260	2.0	7070±110	7370±110	+ 4.2	4
	Dodecyl sulphate	0.160	3.8	7120±240	7070±260	− 0.7	10
	Tetradecyl sulphate	0.060	4.4	7070±150	7070±310	0.0	4

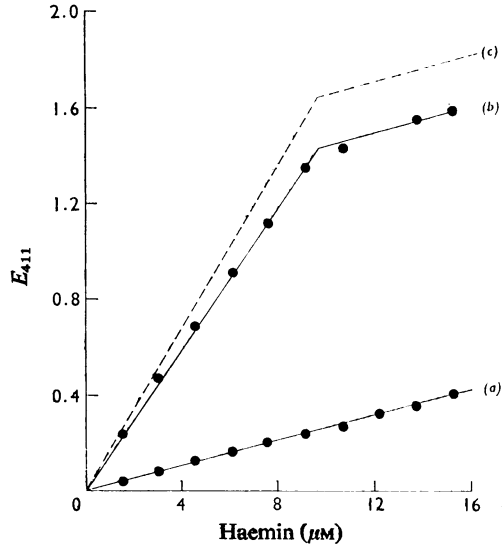

Fig. 6. *Spectrophotometric titration of apomyoglobin with haemin*

The experiment was carried out in 0.033 M-phosphate buffer (pH 7.0). Curve (*a*) shows the absorption of haemin alone, and curve (*b*) the extinction in the presence of 10.0 μM-apomyoglobin. Curve (*c*) represents the theoretical titration curve for full reconstitution, as calculated from the absorption at 411 nm of the metmyoglobin preparation used for preparation of apomyoglobin.

$8\times10^3\,M^{-1}$ under the conditions of the experiment, i.e. a decrease in the association constant of approximately sixfold compared with that in the absence of Bromophenol Blue.

Spectroscopic studies

The general conclusion to be drawn from the competition experiments in the previous section is that, except for protoporphyrin IX, two different ligands may be bound simultaneously by apomyoglobin. It should be noted that the adherence of Bromophenol Blue and protoporphyrin IX to a competitive scheme does not necessarily imply that the two ligands compete for binding at the same site. It could simply mean that Bromophenol Blue and protoporphyrin IX are bound to each of two distinctly different conformations of the protein. Evidence for conformational changes accompanying binding was sought by optical-rotatory-dispersion (Table 3) measurements. In agreement with previous studies (Harrison & Blout, 1965; Breslow *et al.*, 1965) the reduced mean residue rotation at 233 nm ($[m']_{233}$) of apomyoglobin (7120±220, $n = 39$) was smaller than that of metmyoglobin (9310±410, $n = 9$). As indicated by the results in Table 3 a partial reversal of the Cotton trough at 233 nm towards that of native metmyoglobin was observed by addition of haemin to apomyoglobin. Smaller effects on $[m']_{233}$ were generally observed in the presence of the ligands under present study, suggesting no drastic changes in the conformation of the protein. However, the rather high increase in the laevorotation of apomyoglobin by addition of protoporphyrin IX may indicate that the protein acquires some of the features characteristic of the conformation of metmyoglobin by interaction with this ligand.

In contrast with the present results a complete reversal of the optical-rotatory-dispersion curve of apomyoglobin combined with haemin towards that of native metmyoglobin has been reported (Harrison & Blout, 1965; Breslow *et al.*, 1965). However, Breslow *et al.* (1967) indicate that the circular-dichroism spectrum of apomyoglobin plus haemin is intermediate between that of apomyoglobin and native metmyoglobin. We have examined the spectral properties of apomyoglobin plus haemin further by measuring the light absorption of haemin at 411 nm in an apomyoglobin preparation as a function of haemin concentration (Fig. 6). Apomyoglobin

combines with haemin in stoicheiometric amounts, but the absorption increase resulting from the interaction is less than that of native metmyoglobin. It can be calculated from Fig. 2 of the paper of Breslow *et al.* (1965) that the absorption increase of reconstituted metmyoglobin in their experiments was somewhat below that of native metmyoglobin. Therefore the combination of apomyoglobin with haemin was not quite a reversible process in these experiments.

Discussion

Ligand binding by apomyoglobin

The present study shows that apomyoglobin, in contrast to myoglobin, binds with a high affinity a variety of substances. These unphysiological ligands are presumably bound at, or near, the same assembly of non-polar amino acid residues that is in contact with haem in myoglobin. The enhanced binding of alkyl sulphates observed by an increase of the hydrocarbon length of aliphatic compounds (Table 1) attests to the important role of hydrophobic interactions for binding, as does the fact that the free-energy changes accompanying binding is mainly accounted for by entropy changes (Table 2). Thus the binding energy appears to arise primarily from the gain in entropy of water molecules as a consequence of the binding process (the hydrophobic effect), but other kinds of interactions presumably contribute to the binding affinity. As for serum albumin (Steinhardt & Reynolds, 1969) dodecyl sulphate is more strongly bound than dodecanoate by apomyoglobin. This difference may be attributed to the existence of ionic interactions of dodecyl sulphate with proteins (Tanford, 1972).

The size and conformation of the binding site of apomyoglobin appears to change according to experimental conditions as evidenced by the following findings. (1) Four molecules of dodecyl sulphate or tetradecyl sulphate were bound with a high affinity by apomyoglobin without evidence of positive co-operative interaction or major conformational changes in the protein. In contrast, only two molecules of decyl sulphate and dodecanoate were capable of high-affinity interaction with the protein. Hence the available binding area in these cases appears to be determined by the strength of the association rather than by the size of the ligands. (2) Evidence was obtained for concomitant binding of Bromophenol Blue and bilirubin or dodecanoate (Figs. 4 and 5), indicating that these compounds when singly bound do not use up all the binding area. On the basis of these observations it seems likely that the binding sites do not pre-exist, but are moulded from predominantly non-polar amino acid residues by reaction with hydrophobic ligands. A similar model of flexible binding sites has been proposed to account for the adaptability of serum albumin for binding of ligands of a diverse nature (Karush, 1954; Spector, 1975).

For Bromophenol Blue and protoporphyrin IX inhibition of dye binding followed a strictly competitive scheme (Fig. 3). This observation indicates that the protoporphyrin–apomyoglobin complex, like metmyoglobin, does not bind Bromophenol Blue with a high affinity. This finding is not adequately accounted for by exhaustion of the binding area by protoporphyrin IX, since binding of Bromophenol Blue may occur in the presence of bilirubin which has a similar size. Rather it is probable that binding of protoporphyrin IX induces a conformational change which may be more similar to that of metmyoglobin than for the other ligands. In this connexion it is noteworthy that the laevorotation of the apoprotein increased more by interaction with protoporphyrin IX than by binding of the other ligands. It may be that, as in metmyoglobin, the binding site after combination with protoporphyrin IX is removed to a more interior position in the protein structure, which is unavailable for reaction with other ligands.

Comparison between binding ability of apomyoglobin and serum albumin

As mentioned in the introduction, the present study was initiated with a view to obtaining an additional insight into the binding ability of serum albumin. In Table 4 we have assembled selected literature on the interaction of human serum albumin with the same ligands as those studied in the present work (except protoporphyrin IX, for which no characterization of interaction with serum albumin could be found). Accurate results on association constants and number of binding sites on serum albumin are difficult to obtain, owing to the heterogeneity of the binding sites. The data on binding of aliphatic compounds have been taken from a single study to provide a set of internally consistent results. It may be noted that for dodecanoate the more recent results published by Spector (1975) give somewhat lower values for the association constants. A comparison of Tables 1 and 4 shows that in most cases the binding capacity of serum albumin and apomyoglobin is similar when it is taken into account that the mass of serum albumin is about four times larger than that of apomyoglobin. However, notable exceptions are provided by dodecyl sulphate and tetradecyl sulphate, which interact with apomyoglobin to a higher extent. Therefore the total hydrophobic area available for binding by serum albumin in the native state appears to be smaller than that of apomyoglobin. Nevertheless, the association constants of serum albumin for all of the aliphatic

Table 4. *Number of high-affinity binding sites (n) and association constants (K) of various ligands bound by human serum albumin*

Binding of the aliphatic compounds was measured by equilibrium dialysis ($I = 0.033$, 2°C) and binding of Bromophenol Blue by ultrafiltration ($I = 0.075$–0.275, 25°C). The concentration of free bilirubin in the presence of serum albumin was measured on the basis of the rate of oxidation of bilirubin by peroxidase ($I = 0.16$, 37°C). Binding curves of the aliphatic compounds have been reported not to be affected by a change of pH from 5.6 to 7.5 and by a change of temperature from 2°C to 25°C (Steinhardt *et al.*, 1971).

Ligand	pH	*n*	$10^{-6} \times K$ (M^{-1})	References
Decyl sulphate	5.6	8	0.26	Steinhardt *et al.* (1971)
Dodecyl sulphate	5.6	8	2.2	Steinhardt *et al.* (1971)
Tetradecyl sulphate	5.6	9–10	8.6–6.7	Steinhardt *et al.* (1971)
Dodecanoate	5.6	2	1.6	Goodman (1958)
		5	0.24	Steinhardt *et al.* (1971)
Bromophenol Blue	7.0	2	3–15	Kragh-Hansen & Møller (1974)
		3	0.06	
Bilirubin	7.4	1	140	Jacobsen (1969)
		2	0.5	

compounds are about one order of magnitude higher than those of apomyoglobin.

The bottom part of Table 4 shows that the association constants of Bromophenol Blue and bilirubin at the different binding sites of serum albumin are quite variable. In particular it is clear by reference to Table 1 that a special mechanism must account for the binding of the first molecule of bilirubin by serum albumin. In this connexion it may be noted that Lovrien & Sturtevant (1971) showed by calorimetric studies that binding of an equimolar amount of dodecyl sulphate is accompanied by a large enthalpy change (−75 kJ/mol). This finding presumably indicates the existence of one particularly strong binding site for dodecyl sulphate, which has escaped notice in the equilibrium-dialysis experiments. The high enthalpy changes of this site suggests that forces other than hydrophobic interactions are predominant in this case. A major enthalpy change is also associated with the binding of tryptophan by serum albumin (Fairclough & Fruton, 1966). Apart from such cases of specific interactions as mentioned above, the general characteristics of serum albumin binding resemble those of apomyoglobin. Apomyoglobin appears to have a larger hydrophobic area available for high-affinity binding than does serum albumin, and this circumstance, together with the lower association constants for aliphatic compounds, suggest that forces other than hydrophobic interactions are operative in ligand binding by serum albumin. It is possible that the extra binding affinity of serum albumin is caused by the presence of positively charged groups in the vicinity of the binding sites, since this protein combines more strongly with anionic than with neutral (Reynolds *et al.*, 1968) and cationic (Nozaki *et al.*, 1974) compounds.

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