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# The Interaction of Myoglobin with Mitochondrial Structural Protein*

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ABSTRACT: The binding of myoglobin to the structural protein from beef heart mitochondria has been followed by fluorescence titration. A one–one complex is formed between the two proteins with an average dissociation constant of $4.2 \times 10^{-7}$ for metmyoglobin and $8.6 \times 10^{-7}$ for oxymyoglobin.

The $\Delta H$ of dissociation of the complex is $0 \pm 100$ cal/mole and $\Delta S$ is $-29.4 \pm 0.3$ eu. Increasing ionic strengths up to 1.0 M NaCl have no effect on the complex formation.

In studying the specificity of interaction between the structural protein of beef heart mitochondria and the cytochromes, a single nonmitochondrial protein was found to complex stoichiometrically with the structural protein (Criddle *et al.*, 1962). It was demonstrated that myoglobin, but not hemoglobin, could participate in a 1:1 complex which solubilized the structural protein and allowed investigation of the physical properties of this protein preparation. The significance of this interaction was suggested from the observations that (a) heart mitochondria as normally isolated are contaminated by myoglobin which can be removed only by extensive washing; and (b) a direct relationship exists between the efficiency of respiration of muscle tissue and the myoglobin concentration of that tissue (Lawrie, 1953). This suggests a possible direct role of myoglobin in transporting oxygen to the mitochondria, and thereby increasing the local oxygen concentration at the site of oxygen utilization.

This interaction between structural protein and myoglobin has now been studied in greater detail using the method of fluorescence quenching to determine the binding constant, the stoichiometry, and the homogeneity of binding sites involved in the interaction.

## Methods and Materials

Metmyoglobin was purchased from Calbiochem, Los Angeles, Calif. It was used without further purification. Ten–twenty milligrams was routinely dissolved in 0.005 M Tris–chloride buffer pH 9.0 to give a concentration of 1 mg/ml. Exact concentrations were determined from the optical density at 630 mμ (Theorell, 1934); this solution was used directly for fluorescence titration.

Oxymyoglobin was prepared from beef heart by a modification of the method of Yamazaki *et al.* (1964) which described its preparation from horse heart. Only the chromatography procedure was modified. Chromatography was carried out on DEAE-cellulose, prepared as described by Peterson and Sober (1962), which was equilibrated with 0.005 M Tris–HCl buffer pH 8.4. The cellulose was packed into a column ($2 \times 80$ cm) under 4 psi air pressure. The oxymyoglobin preparation was applied to the column at 5° using slight pressure. The column was then washed for 2 hr with 0.005 M Tris–HCl buffer pH 8.4 at a flow rate of 30–40 ml/hr. Oxymyoglobin was eluted from the column using a stepwise elution procedure. A change in the buffer to 0.05 M Tris–HCl pH 8.4 resulted in the elution of first metmyoglobin and then oxymyoglobin. A second fraction containing only oxymyoglobin was eluted with 0.05 M Tris–chloride buffer with 0.2 M NaCl. The presence of met- or oxymyoglobin was determined by measuring the absorption spectrum of the eluted protein between 400 and 650 mμ (Yamazaki, 1964).

Mitochondrial structural protein was prepared by the method of Richardson *et al.* (1963) and solubilized as described previously (Edwards and Criddle, 1966). Two milliliter samples of structural protein containing 0.1 mg of protein/ml were titrated with 0.01-ml aliquots of the preparations of heme protein. The data from the titration curves was analyzed using an IBM 7040 computer.

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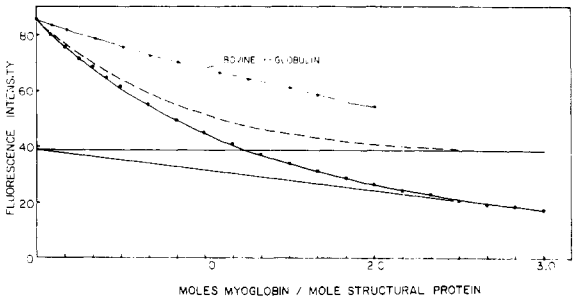


FIGURE 1: Fluorescence titration of mitochondrial structural protein with myoglobin. A control experiment is shown in which bovine γ-globulin is substituted for structural protein. No quenching of fluorescence is observed. A titration of structural protein with hemoglobin also shows no quenching.

TABLE I: Dissociation Constants and Free Energies Calculated from the Structural Protein–Metmyoglobin Curve.

| ml × $10^{2a}$ | Dissociation Constant × $10^{7}$ | Free Energy (cal/mole × $10^{-3}$) |
|---|---|---|
| 2 | 0.76 | 9.72 |
| 3 | 2.14 | 9.11 |
| 4 | 2 95 | 8.92 |
| 5 | 3.82 | 8.76 |
| 6 | 4.36 | 8.68 |
| 7 | 4.88 | 8.62 |
| 8 | 5.06 | 8.60 |
| 9 | 5.26 | 8.57 |
| 10 | 5.42 | 8.56 |
| 11 | 5.56 | 8.54 |
| 12 | 5.61 | 8.54 |
| 13 | 5.30 | 8.57 |
| 14 | 5.09 | 8.59 |
| 15 | 4.84 | 8.62 |
| 16 | 4.68 | 8.64 |
| 17 | 4.44 | 8.67 |
| 18 | 4.26 | 8.70 |
| 19 | 3.94 | 8.74 |
| 20 | 3.68 | 8.79 |
| 21 | 3.22 | 8.86 |
| 22 | 2.85 | 8.94 |
| Average | 4.19 | 8.75 |

[a] Milliliters of metmyoglobin.

## Results

A curve for the titration of structural protein with metmyoglobin is shown in Figure 1. The end point of the titration, found at the intercept of the extrapolated initial and final slopes of the curve, indicates a 1:1 stoichiometry for the reaction. Control curves obtained by the substitution of hemoglobin for myoglobin or bovine γ-globulin for structural protein show no binding. Plotting the data by the method of Scatchard (1959) also indicates a 1:1 stoichiometry (see Figure 2). The intercept on the $N$ axis gives the number of binding sites for myoglobin per molecule of structural protein. The intercept was found to be 1 ± 0.2 for several experiments.

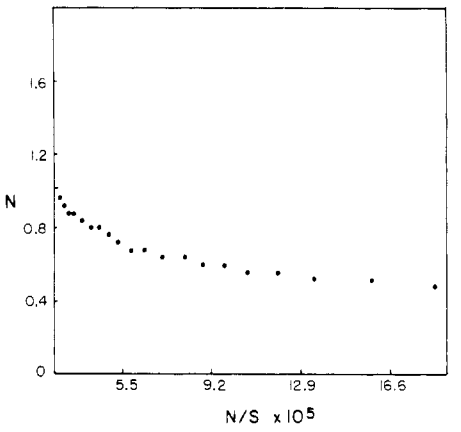


FIGURE 2: A Scatchard plot of the data from the myoglobin titration of structural protein. $N$ is moles of myoglobin bound/mole of structural protein. $S$ is moles of free myoglobin. The intercept on the $N$ axis indicates that 1 mole of myoglobin is bound per mole of structural protein.

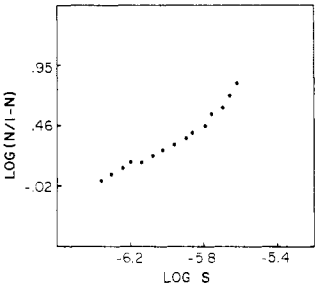


FIGURE 3: Heterogeneity plot for myoglobin binding to structural protein. $N$ and $S$ are as in Figure 2. The least-squares slope of the line is 0.96, indicating a narrow distribution of binding energies.

Table I shows the dissociation constants and free energies of binding calculated for experimental points on the titration curve of Figure 1. Concentrations of free structural protein, free myoglobin, and complex were determined from the titration curve for use in these calculations (Edwards and Criddle, 1966).

Figure 3 shows the data from the titration curve plotted by the method of Sips (1948). The least-squares slope of the line is 0.96, indicating a narrow distribution of binding energies. A plot of complex formation as a function of added metmyoglobin (Edwards and Criddle,

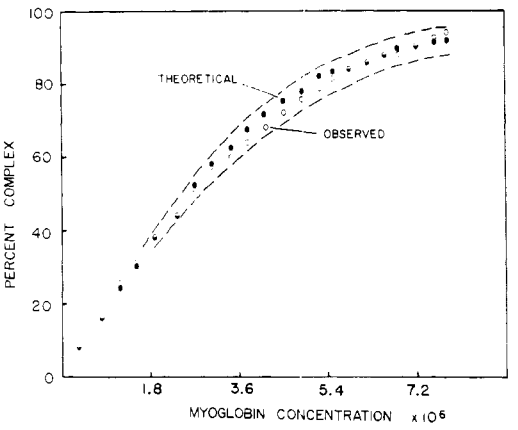


FIGURE 4: A plot of complex formation as a function of added myoglobin. The "theoretical" values obtained from the average dissociation constant are compared with experimentally obtained values from the titration curve. The dashed lines indicate ±4% deviation in the average value of $\Delta F$ of dissociation.

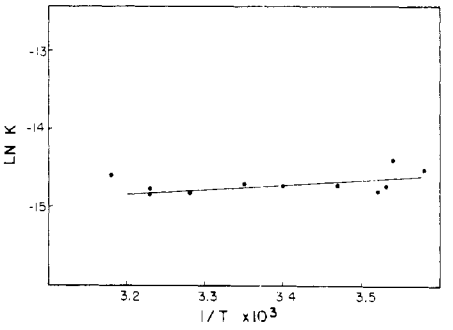


FIGURE 5: Temperature dependence of the dissociation constant of the myoglobin–structural protein complex. $T$ is absolute temperature and $K$ is the dissociation constant.

1966) is shown in Figure 4. A "theoretical" curve calculated from the average dissociation constant is compared with observed values from the experimental curve. The closeness of fit of the two curves indicates that the experimental curve can be approximated by a single dissociation constant. The sensitivity of the curve of average dissociation constant to a ±4% change in $\Delta F$ of dissociation is indicated by the dashed lines.

An Arrhenius-type plot for obtaining enthalpy and entropy values for the interaction is shown in Figure 5. The absence of any appreciable slope to the curve indicates that the enthalpy contribution to the interaction is very small. At 25°, the $\Delta H$ of dissociation, is calculated to be 0 ± 100 cal; $\Delta S$ is, therefore, −29.4 ± 0.3 eu.

Table II shows that little effect on the binding of myoglobin by structural protein interaction is observed with an increase in ionic strength by addition of NaCl.

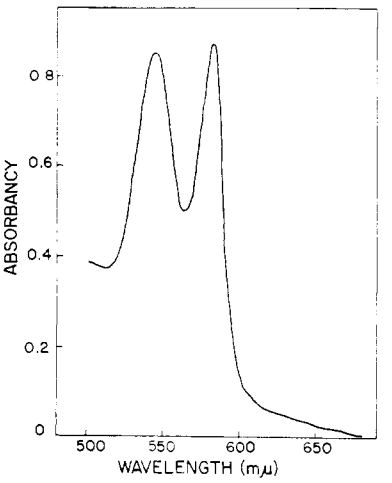


FIGURE 6: Absorption spectrum of oxymyoglobin.

Salt concentrations were varied over the range 0–1 M in NaCl with no change in the binding energy.

All of the structural protein molecules are able to bind myoglobin. The site of binding is lost upon oxida-

TABLE II: Effect of Ionic Strength on the Dissociation of the Metmyoglobin–Structural Protein Complex.

| NaCl (M) | Stoichiometry of Binding | Dissociation Constant × $10^7$ |
|---|---|---|
| 0.0 | 1:0.93 | 4.0 |
| 0.1 | 1:1.22 | 3.5 |
| 0.2 | 1:1.06 | 4.1 |
| 0.5 | 1:1.15 | 3.1 |
| 1.0 | 1:0.92 | 4.2 |

TABLE III: The Relation between SH Content of Structural Protein and Myoglobin Binding.

| Sample No. | SH[a] | % Binding[b] |
|---|---|---|
| Mb 1 | 0.78 | 85 |
| Mb 2 | 0.62 | 68 |
| Mb 3 | 0.32 | 29 |
| Mb 3[c] | 0.87 | 89 |

[a] Moles of free sulfhydryl per 22,000 g of structural protein determined by the method of Ellman (1959). [b] Determined from fluorescence quenching curve as described by Edwards and Criddle (1966). [c] Dialyzed *vs.* Tris–HCl buffer 0.001 M, pH 9, and incubated with 0.001 M dithioerythritol before measurement.

tion of a single sulfhydryl residue which yields a dimerized, disulfide cross-linked, structural protein. These results are identical with those found for cytochrome *c* (Criddle *et al.*, 1966). (See Table III.)

Data obtained from the titration curve of structural protein with completely reduced, oxygenated myoglobin is presented in Table IV. An absorption spectrum of the oxymyoglobin preparation used in these experiments is shown in Figure 6. Stoichiometry and heterogeneity of binding measurements yield results nearly identical with those of metmyoglobin. The dissociation constant observed for the reaction is larger, however, indicating that oxymyoglobin is bound less tightly to the structural protein than is metmyoglobin.

TABLE IV: Data for the Binding of Oxymyoglobin to Structural Protein.

| | |
|---|---|
| Stoichiometry | 1:1.14 |
| Dissociation constant | $8.59 \times 10^{-7}$ |
| Free energy (cal/mole) | $8.44 \times 10^{3}$ |
| Per cent of structural protein binding oxymyoglobin[a] | 85.0 |

[a] See Edwards and Criddle (1966).

## Discussion

The structural protein from mitochondria was first characterized by its ability to interact with itself to form high polymers, with mitochondrial lipids, and with the cytochromes to form protein complexes. In tests with a group of 18 nonmitochondrial proteins, only myoglobin was found to be capable of forming a soluble, stoichiometric complex (Criddle *et al.*, 1962). This binding specificity was also noted in tests of the somewhat more limited interaction of soluble proteins with suspensions of insoluble structural protein suspensions (Criddle *et al.*, 1961). The solubilization process, therefore, does not seem to affect the specificity of the interaction. A second question that may be asked, however, is whether the solubilization process with its concomitant formation of complex of SDS and protein has a marked effect on the stability of the protein complexes formed between myoglobin or cytochromes and structural protein. This question is as yet unanswered and the data must, therefore, be interpreted in terms of this limitation.

Oxymyoglobin is less tightly bound by the solubilized structural protein than is the unoxygenated metmyoglobin form. While it is not yet known whether this difference in binding is due to the reduction of the heme iron or to the presence of the oxygen molecule, the difference suggests a change in the myoglobin structure or properties which affects the binding region. Any mechanism which can effectively increase the local concentration of oxygen at the mitochondria, the major site of oxygen utilization, would be of physiological advantage to the organism. This could be accomplished either by an increased affinity of oxymyoglobin for the mitochondrial membrane over that of the unoxygenated form or by an increased affinity of mitochondrial bound myoglobin for oxygen over that of free myoglobin in the presence of a mechanism for transporting oxygen from the bound myoglobin through the mitochondrial membrane. Both of these possibilities will require further investigation.

Complex formation has been studied over a range of protein concentrations, and the thermodynamic values are independent of the concentrations of either species. The thermodynamic values obtained for the binding indicate that the complex is predominantly entropy stabilized, and it has been shown that the reaction is nearly insensitive to concentration of added salt. The salt effect is in marked contrast to that noted for the cytochrome *c*-structural protein complex which is readily dissociated by 0.1 ionic strength sodium chloride. In spite of the differences noted for the effect of salt on the interactions of the two heme proteins, there is a great similarity noted between the thermodynamic parameters for the reactions.

The formation of a complex containing more than one of the cytochromes per structural protein molecule, or a cytochrome plus myoglobin per structural protein molecule, has not been possible (Criddle *et al.*, 1962). The binding site for both cytochrome *c* and myoglobin is lost upon dimerization of structural protein through the formation of a single disulfide bond between the structural protein monomers and can be regained by reduction of this bond. This behavior suggests a possible overlap or identity of the region on the structural protein molecule involved in the binding of the two heme proteins.

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