Binding of Cytochrome *c* by Mitochondrial Structural Protein*

D. L. Edwards† and R. S. Criddle

ABSTRACT: The reaction of cytochrome *c* with the structural protein from beef heart mitochondria has been followed by the procedure of fluorescence quenching. In pH 9, 0.005 M Tris chloride buffer, the dissociation constant for the reaction for formation of a 1:1 complex of these proteins has been determined to be 4.1×10^{-7}. Temperature dependence of the dissociation constant indicates a value of $\Delta H = 0 \pm 100$ cal/mole and ΔS of -30.4 ± 0.4 eu. The interaction is markedly dependent on ionic strength, with NaCl concentrations of 0.1 M dissociating the complex completely. The titration curve for the reaction between cytochrome *c* and structural protein can be closely described using a single dissociation constant.

The structural protein (SP[1]) from beef heart mitochondria has been studied physically and chemically and found to be a single homogeneous chemical species (Criddle *et al.*, 1962, 1966). This protein has been shown to react with cytochromes *b*, *a*, and c_1 to form specific stoichiometric complexes which have been studied using ultracentrifugation and electrophoresis. These studies failed to demonstrate any interaction between the structural protein and cytochrome *c*. The absence of interaction between these two species was rationalized on the basis of ease of extractability of cytochrome *c* from the mitochondrion with procedures such as dilute saline extraction.

We have reinvestigated the possible interaction of cytochrome *c* and structural protein using the much more sensitive method of fluoresence quenching. With this procedure, the thermodynamics of the interaction have been studied and it has been demonstrated that the effect of ionic strength on the interaction was responsible for the earlier failure to detect this complex formation.

Materials and Methods

Cytochrome c. Horse heart cytochrome *c* type III was purchased from Sigma Chemical Co., St. Louis, Mo., and was used without further purification. Ten to twenty mg of protein was routinely dissolved in 0.005 M Tris–HCl buffer pH 9.0 to give a concentration of 1 mg/ml. Exact concentrations were determined from optical density at 550 mμ (Paleus and Paul, 1963). This solution was then used directly for titrations.

Mitochondrial Structural Protein. Beef heart mitochondria were prepared by the method of Beinert (1957). Structural protein (SP)[1] was isolated from these mitochondria by the method of Richardson *et al.* (1963) which included disruption of the mitochondria with cholate and deoxycholate, ammonium sulfate fractionation, and acetone extraction to remove lipid and bile acids. The protein was solubilized by adjusting the pH of a 1-mg/ml slurry to 10.5 and adding 0.1 mg/ml of two times recrystallized sodium dodecyl sulfate. The protein was solubilized during 5–6 hr stirring at room temperature. The solution was then dialyzed overnight in the cold against 0.005 M Tris–HCl pH 9.0. Insoluble material was removed by centrifugation at 20,000 × g for 10 min. Concentrations were determined from the optical density at 280 mμ (Criddle *et al.*, 1962). The solution was stored at −20°.

Fluorescence Titration. Fluorometric titrations were carried out in an Aminco-Bowman spectrophotofluorometer which had been modified to contain a thermostatically controlled cuvet holder. The contents of the cuvet were stirred continuously using a water-driven magnetic stirrer which was mounted on the bottom of the cell housing. A 21-gauge Teflon syringe needle was passed through the wall of the cell housing and into the cuvet which was fitted with a plastic cap. The tip of the syringe needle was allowed to extend below the surface of the liquid in the cuvet but did not extend so far as to be in the light path. Cytochrome *c* (1 mg/ml) was added to the cuvet by means of an 0.5-ml micrometer syringe which was purchased from Burroughs-Wellcome Co., Liverpool, England.

Samples containing 0.1–0.5 mg of structural protein in 2.0 ml of 0.001 M Tris–Cl pH 9.0 were routinely titrated. Before each titration the sample of structural protein was incubated for 10 min in the instrument to allow thermal equilibration. Cytochrome *c* was added in 0.01-ml aliquots. It was found that a period of 1 min between additions was sufficient for equilibrium to be attained. Protein fluorescence was activated with incident light at 290 mμ and emission was read at 350 mμ. Samples were shielded from incident light except when readings were being taken. A plot of fluorescence intensity *vs.* concentration of added cytochrome *c* was made for each titration.

*From the Department of Biochemistry and Biophysics, University of California, Davis, California. *Received July 6, 1965; revised October 13, 1965.* This research was supported in part by a grant (GM 10017) from the U. S. Public Health Service.

† Predoctoral Fellow of the U. S. Public Health Service.

[1] Abbreviation used: SP, structular protein.

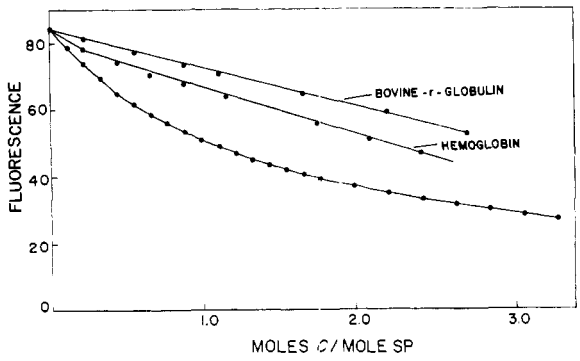

FIGURE 1: A titration curve for structural protein–cytochrome *c*. Structural protein is placed in the cuvet and is titrated with cytochrome *c*. A control titration substituting bovine γ-globulin for structural protein shows no quenching of fluorescence but only a constant slope due to beam attenuation. A control experiment substituting hemoglobin for cytochrome *c* is also shown.

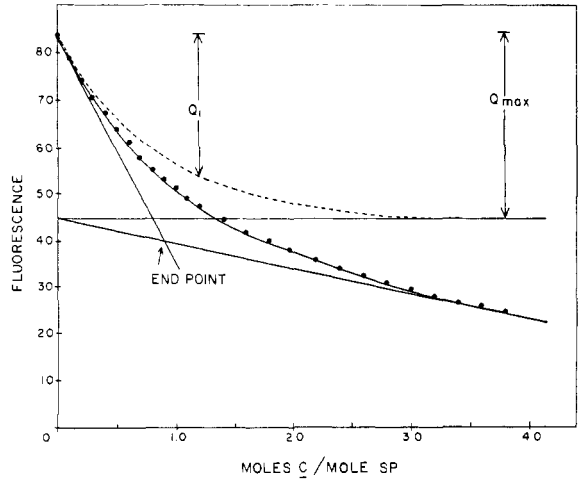

FIGURE 2: Correction of the titration curve for beam attenuation. The experimental curve (filled circles) is corrected for beam attenuation as described by Velick (1960). The corrected curve (dashed line) is then used to obtain the values Q_i and Q_{max}.

Treatment of Data

Plots of fluorescence intensity *vs.* cytochrome *c* concentration were corrected for beam attenuation as described by Velick *et al.* (1960). Complex formation was determined for each experimental point, as described by Eisen and Siskind (1964), where the amount of complex is calculated from the degree of quenching observed (Q_i) relative to the quenching when all binding sites are occupied (Q_{max}). The amount of free cytochrome *c* is calculated as the difference between added and bound *c*. Experimentally determined values of (Q_i), (Q_{max}), temperature, ionic strength, cytochrome *c*, and structural protein concentrations were read into an IBM 7040 computer which performed all calculations.

Correction for Nonbinding Protein. A correction for nonbinding structural protein was made by considering the initial portion of the titration curve. As the dissociation constant of the structural protein–cytochrome *c* complex is small, it can be assumed that in the region of large structural protein excess, *i.e.*, the first portion of the titration curve, all added cytochrome will be bound. With a 1:1 reaction stoichiometry, an equal number of structural protein molecules will also be bound. In this case, correction was made for nonbinding material using the approximation that for the first three additions of cytochrome *c* (0.03 ml) all of the cytochrome is bound. This value, when compared with the amount of complex formed as determined from the experimental curve, permits calculation of the fraction of protein capable of binding. The correction factor for nonbinding protein was determined from each of the first three additions of cytochrome, and the average of three values was used. The validity of this approximation can readily be obtained through calculation of the amount of free cytochrome *c* expected at these concentrations based on the observed dissociation constant.

Stoichiometry. The end point of the titration is found at the intersection of the extrapolated initial and final slopes of the titration. This gives the reaction stoichiometry directly. The stoichiometry can be checked by using the method of Scatchard (1959). A plot of N *vs* N/S, where N is moles of cytochrome *c* bound per mole of structural protein and S is moles of free cytochrome, will extrapolate to an intercept on the N axis that is equal to the number of cytochrome *c* binding sites per mole of structural protein. The slope of the curve is equal to $-K_{dis}$, the dissociation constant. The slope and N-intercept of each plot were determined by the method of least squares.

Dissociation Constants. Having established the stoichiometry of the reaction, a dissociation constant was calculated for each experimental point on the titration curve. Concentrations of free cytochrome *c*, free structural protein, and complex were calculated at points corresponding to each 0.01-ml addition of *c*. A dissociation constant was determined for each point on the curve using the relationship

$$K_{dis} = \frac{(c - x)(SP - x)}{(x)}$$

where c = cytochrome *c*, SP = structural protein, and x = complex. All values are in moles/liter.

An average dissociation constant was calculated from the values obtained at each point on the titration curve. This value was then used in generating a "theoretical" curve which one would obtain if the reaction forming the protein complex could be described by a single dissociation constant. Comparison of the curve plotted for a single dissociation constant with that obtained experimentally can then be used as an indication of the

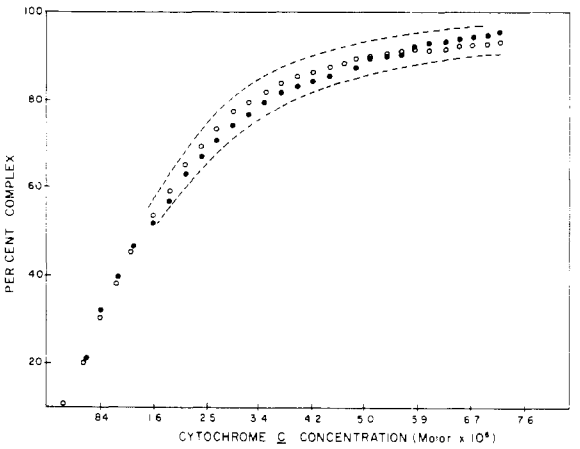

FIGURE 3: Plot of structural protein–cytochrome *c* complex formation as a function of added cytochrome *c*. Complex formation is complete when all of the binding sites for cytochrome *c* on the structural protein are filled. Values calculated from the experimental curve (filled circles) are compared with "theoretical" values calculated using the average dissociation constant (open circles). The dashed lines indicate a deviation of $\pm 4\%$ of the ΔF of the reaction.

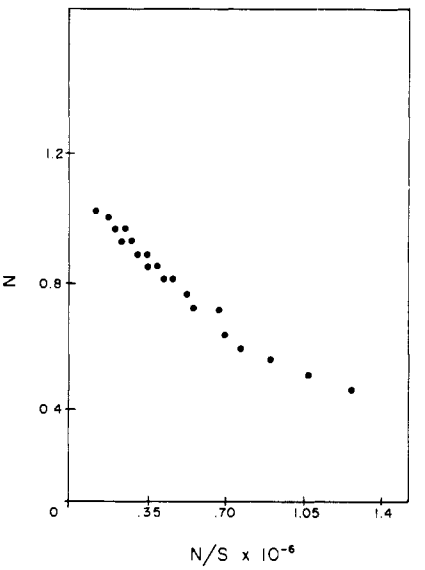

FIGURE 4: Data from the structural protein–cytochrome *c* titration curve plotted by the method of Scatchard. The intercept on the *N* axis, obtained from extrapolation of the least-squares-determined slope, represents the number of binding sites for cytochrome *c* per mole of structural protein. This value was found to be 1 ± 0.2 in replicate experiments. *N* is moles of cytochrome *c* bound per mole of structural protein. *S* is moles of free cytochrome.

distribution of binding energies of the molecules involved in the reaction.

Heterogeneity. The distribution of binding energies was studied by the method of Sips (1948) (see Eisen and Siskind, 1964). A heterogeneity constant was calculated from the slope of the curve obtained by the method of least squares. The heterogeneity constant is indicative of the distribution of dissociation constants about the average dissociation constant. If equal to 1, there is but a single dissociation constant. Decreasing values for the heterogeneity constant represent increasing heterogeneity of binding.

Results

Binding of Cytochrome c to Mitochondrial Structural Protein. A typical titration curve of mitochondrial structural protein with cytochrome *c* is shown in Figure 1. The experimental curve is corrected for beam attenuation as is shown in Figure 2. The values Q_i and Q_{max} are determined from the corrected curve. Controls using bovine γ-globulin in place of structural protein and hemoglobin substituted for cytochrome *c* show no fluorescence quenching. Table I summarizes the dissociation constants, free energies, and binding data of the experiment. The correction for nonbinding material shows that in this experiment 72.7% of the structural protein binds cytochrome *c*. This value has varied from 20 to 95% with various preparations of structural protein (Criddle *et al.*, 1966), and varies with the age of the preparation due to intermolecular disulfide bond formation. In general, freshly prepared samples of structural protein have 60–90% binding. The dissociation constant for the actively binding protein does not change with change in per cent binding, however. Figure 3 is a plot of the per cent of structural protein existing as complex as a function of the concentration of added cytochrome *c* given for both the experimentally observed points and for the "theoretical" points calculated from the average dissociation constant. It can be seen that the experimental curve can be quite well approximated using a single dissociation constant. The sensitivity of the curve is such that a deviation of $\pm 4\%$ in ΔF would readily be detectable.

Stoichiometry. The end point of the titration is shown in Figure 2 to indicate a 1:1 stoichiometry for the structural protein–cytochrome *c* complex. A Scatchard type plot of these same data is shown in Figure 4. The intercept on the *N* axis again indicates a 1:1 stoichiometry as values of the intercept were found to be 1.0 ± 0.2 in repeated experiments. The dissociation constant calculated from the slope of Figure 4 agrees well with the average dissociation constant from the titration curve.

Heterogeneity. A heterogeneity plot using the method of Sips (1948) is shown in Figure 5. The heterogeneity index calculated from the slope is 0.89, indicating a relatively narrow distribution of binding energies. A dissociation constant may be calculated from the intercept using this method and gives a value which agrees well with other methods of calculation.

Thermodynamic Parameters. An Arrhenius-type plot

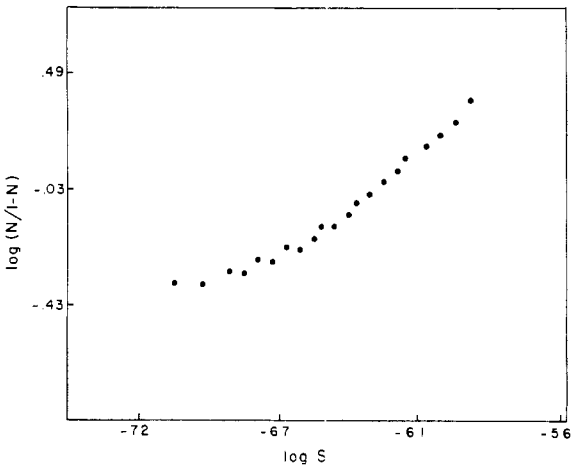

FIGURE 5: The data from the structural protein–cytochrome *c* titration curve plotted by the method of Sips. The slope of the line yields a heterogeneity index indicating the distribution of dissociation constants about the average dissociation constant. *N* is moles of cytochrome *c* bound per mole of structural protein. *S* is moles of free cytochrome *c*.

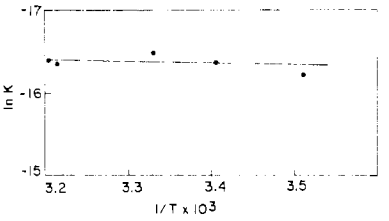

FIGURE 6: An Arrhenius-type plot to determine thermodynamic parameters. Ln *K* is the natural logarithm of the average dissociation constant. *T* is absolute temperature.

of the dissociation constant as a function of temperature is shown in Figure 6. The absence of any appreciable slope for this curve indicates a small enthalpy contribution. ΔH was calculated to be 0 ± 100 cal. The value of ΔS is, therefore, -30.4 ± 0.4 eu.

Effect of Ionic Strength on Complex Formation. Figure 7 shows the titration of structural protein with cytochrome *c* both in the presence and absence of 0.1 M NaCl. With NaCl present no fluorescence quenching is observed. Figure 8 shows a titration curve in which cytochrome *c* is replaced by 3 M NaCl after 0.08 ml of titrating solution has been added. The increase in fluorescence which is noted is indicative of the disruption of the complex. Further addition of cytochrome *c* in the presence of NaCl gives no additional binding but results only in beam attenuation.

Discussion

Due to the method employed for following the formation of the *c*–structural protein complex and for calculating the resultant amount of complex formed, some difficulties arise in stoichiometry and, therefore, dissociation constant determination. The Scatchard plot

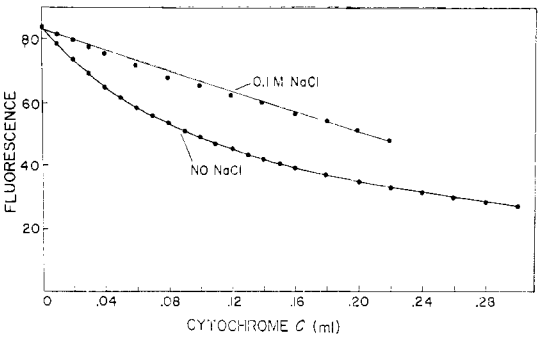

FIGURE 7: Effect of ionic strength on the binding of cytochrome *c* by structural protein. Structural protein is titrated with cytochrome *c* in the presence and absence of 0.1 M NaCl. With NaCl present no fluorescence quenching is observed. In the absence of NaCl a normal quenching curve is seen.

TABLE I: Dissociation Constants and Free Energies Calculated for Experimental Points on the Structural Protein–Cytochrome *c* Titration Curve.

Point No.[a]	Dissociation Constant $\times 10^7$	Free Energy of Dissociation (cal/mole $\times 10^{-3}$)
3	1.94	9.31
4	3.05	9.04
5	3.99	8.87
6	4.48	8.80
7	4.84	8.76
8	4.86	8.74
9	4.87	8.75
10	4.45	8.81
11	4.54	8.80
12	4.44	8.81
13	4.42	8.81
14	4.41	8.82
15	4.33	8.83
16	4.21	8.84
17	4.01	8.87
18	3.91	8.89
19	3.70	8.92
20	3.44	8.96
Average	4.10	8.87

[a] Point numbers refer to 0.01-ml aliquots of cytochrome *c* added, *i.e.*, point number 5 is the point on the curve where 0.05 ml of *c* had been added.

will extrapolate to a correct binding ratio for the cases of the type: n(SP) + cyt. $c \rightleftharpoons$ (SP)$_n$(cyt. c), as long as all the structural protein molecules are bound equally to the cytochrome. In cases SP + n(cyt. c) $\rightleftharpoons$ SP(cyt. c)$_n$, however, in which the first molecule of heme protein going on to the structural protein quenches most of the fluorescence, this treatment of the data will extrapolate to a value of one for the binding ratio even for large values of n. The slope of the plot, and, therefore, the calculated dissociation constant, will be affected in this case. The slope will vary with the value of n and will depend on the relative values of the n association constants for addition of the heme protein to the complex. Stoichiometry determination under these conditions where more than one cytochrome is bound per structural protein molecule must come from inspection of the end point of the titration curve or from other experimental methods. Where the binding of the first cytochrome molecule is much stronger than binding of subsequent molecules, determination of stoichiometry from fluorescence titration curves will not be possible in some cases.

The investigations of the interaction of cytochrome c with structural protein have indicated some of the general characteristics of the structural protein and of the nature of the interactions between structural protein and other mitochondrial components. Since cytochrome c will react stoichiometrically with structural protein, a binding site for cytochrome c must exist on each molecule. It has previously been demonstrated that structural protein will react stoichiometrically with cytochromes c_1, b, and a and also with myoglobin (Criddle *et al.*, 1962; Edwards and Criddle, 1966). A single molecule of structural protein can bind no more than one cytochrome molecule, *i.e.*, it has not been possible to form a complex of SP(cyt. c)$_n$ where $n > 1$, for either one or a mixture of two or more cytochromes. This suggests that a single region on the structural protein may be involved in complex formation with all of the heme proteins which are bound.

Cytochrome c binding, which appears to be largely ionic in nature based on its ease of reversibility with dilute saline solutions, is prevented from complex formation by oxidation of a single sulfhydryl residue to a disulfide bond (Criddle *et al.*, 1966). This same oxidation prevents myoglobin binding although this reaction is insensitive to ionic strength over the region tested. This again suggests coincidence or overlap of the heme protein binding sites.

Cytochrome c interaction with mitochondrial lipids and with lipid micelles has been extensively studied. It has been noted that lipid–cytochrome c complexes which are completely soluble in organic solvents can be formed with titration of the positive charges of cytochrome c. Ready extractability of c from the lipid complexes occurs by use of dilute saline solution, as is the case with intact mitochondria. This observation has suggested to workers that the c–lipid complex is the existent form of c in the mitochondria. Our experiments indicate that using the same logic, similar conclusions can be drawn regarding the c-structural protein complex as the naturally occurring species. It is suggested that, in fact, the cytochrome c attachment to mitochondria may lie in between the two possible extreme conditions of all lipid–protein complex or all protein–protein complex and that both play a role in its physiological organization.

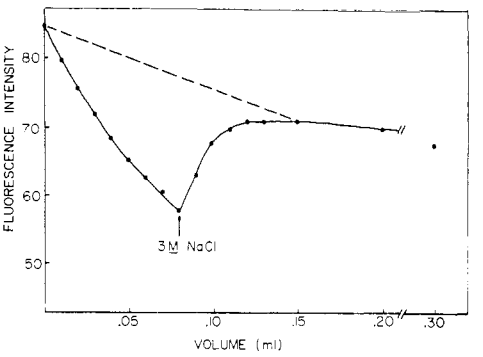

FIGURE 8: Dissociation of the structural protein–cytochrome c complex with increasing ionic strength. Structural protein was titrated with cytochrome c until 0.08 ml of c had been added. At that point cytochrome c was replaced by 3 M NaCl. The dissociation of the complex is observable as an increase in fluorescence. The dashed line indicates the decrease in fluorescence due to beam attenuation. Further addition of cytochrome c after NaCl addition results only in beam attenuation.

Studies on mitochondrial structural protein to date indicate that it is a single protein species; that it binds each of the cytochromes and myoglobin stoichiometrically; and that a single region on the protein functions in the complex formation. This implies that any order existing in the arrangement of cytochromes along the membrane network of the mitochondrion does not have its origin at the level of cytochrome–membrane interaction governed by the formation of these protein complexes.

Acknowledgment

The authors wish to thank Mr. John Griffin for his help in the initial phases of the computer programming.

References

Beinert, H. (1957) *in* Manometric Techniques, Umbreit, W. W., Burris, R. H., and Stauffer, J. F., Ed., Minneapolis, Minn., Burgess, p 143.

Criddle, R. S., Bock, R. M., Green, D. E., and Tisdale, H. (1962), *Biochemistry 1*, 827.

Criddle, R. S., Edwards, D. L., and Peterson, T. G. (1966), *Biochemistry 5*, 578 (this issue; preceding paper).

Edwards, D. L., and Criddle, R. S. (1966), *Biochemistry 5*, 588.

Eisen, H. N., and Siskind, G. W. (1964), *Biochemistry 3*, 996.

Paleus, S., and Paul, K. G. (1963), *Enzymes 8*, 108.
Richardson, S. H., Hultin, H. O., and Green, D. E. (1963), *Proc. Natl. Acad. Sci. U. S. 50*, 821.
Scatchard, G. (1959), *Ann. N. Y. Acad. Sci. 51*, 660.
Sips, R. (1948), *J. Chem. Phys. 16*, 490.
Velick, S., Parker, C. W., and Eisen, H. N. (1960), *Proc. Natl. Acad. Sci. U. S. 46*, 1470.

The Interaction of Myoglobin with Mitochondrial Structural Protein*

D. L. Edwards† and R. S. Criddle

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 4.2×10^{-7} for metmyoglobin and 8.6×10^{-7} for oxymyoglobin.

The ΔH of dissociation of the complex is 0 ± 100 cal/mole and ΔS is -29.4 ± 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 (2×80 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.

* From the Department of Biochemistry and Biophysics, University of California, Davis, California. *Received July 6, 1965; revised October 13, 1965.* This research was supported in part by a grant (GM 10017) from the U. S. Public Health Service.

† Predoctoral Fellow of the U. S. Public Health Service.