Quantitative Demonstration of the Reciprocity of Ligand Effects in the Ternary Complex of Chicken Heart Lactate Dehydrogenase with Nicotinamide Adenine Dinucleotide and Oxalate†

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ABSTRACT: The reciprocity of effects of two ligands simultaneously bound to a protein as a ternary complex may be proven by measurements of four standard free energies of binding. Two of these are for the binding of each ligand in the absence of the other, and the other two for the binding of each ligand in the presence of saturating amounts of the other (conditional free energies). These four quantities have been measured for the complexes of oxalate and nicotinamide adenine dinucleotide with chick heart lactate dehydrogenase. The differences between conditional and unconditional free energies are: oxalate, -1.1 ± 0.3 kcal; NADH, -1.3 ± 0.2 kcal, thus proving the reciprocity within experimental error.

The existence of reciprocal effects between two ligands simultaneously bound to a protein has been repeatedly noticed since the classical observations on the Bohr effect in hemoglobin (Wyman, 1964; Antonini et al., 1963). More recent observations include the reciprocal effects of oxygen and diphosphoglycerate in hemoglobin (Benesch et al., 1968) and those of Mn^{2+} and phosphoenol pyruvate in pyruvate kinase (Nowak and Mildvan, 1972). However, in none of these cases has a rigorous experimental demonstration of the reciprocity of the effects been carried out in quantitative terms.

For the simplest possible case of two interacting ligands X and Y simultaneously bound to a protein P, the quantitative demonstration of reciprocity requires the measurement of four standard free energies of ligand binding. Two of the free energies are those for the binding of one ligand in the absence of the other, $\Delta F°(X)$ and $\Delta F°(Y)$, respectively. The other two free energies correspond to the binding of X to PY and the binding of Y to PX, and are the conditional free energies of binding, $\Delta F°(X/Y)$ and $\Delta F°(Y/X)$, respectively. As we have shown elsewhere (Weber, 1972), free energy conservation requires that

$$\Delta F°(X/Y) - \Delta F°(X) = \Delta F°(Y/X) - \Delta F°(Y) = \Delta F_{xy} \quad (1)$$

where ΔF_{xy} is the free energy coupling between the bound ligands. From the values in the literature (Weber, 1975) we know that ΔF_{xy} is unlikely to exceed 1.5 kcal/mol. To obtain values of ΔF_{xy} accurate to 10–20% requires a determination of the four dissociation constants ($K(X)$, $K(Y)$, $K(X/Y)$, $K(Y/X)$) corresponding to the four free energy values of eq 1 with approximate similar precision. This requirement is not easily met and this partially explains the paucity of relevant data in the literature. However, we are able to present here a case in which we found it possible to measure independently the four constants in question and verify the reciprocity condition with satisfactory accuracy.

Reciprocal Effects of Oxalate and NADH Bound to Lactate Dehydrogenase. Winer et al. (1959) have shown among others that the addition of oxalate to lactate dehydrogenase results in an enhancement both of the fluorescence and the binding free energy of NADH. On the other hand, the exact effect of NADH upon oxalate binding by the dehydrogenase has never been determined. Indeed the statement found in the literature that "oxalate is not bound in the absence of NADH" implies a failure of reciprocity, and indirectly of free energy conservation in the system. For this reason we investigated this case with great care. Chicken heart lactate dehydrogenase was chosen as this enzyme has characteristics of purity and stability that are particularly favorable. Moreover, preliminary experiments on the binding of NADH and its enhancement by oxalate showed completely independent behavior of the four subunits of the protein. This case lacks, therefore, the complexities that are introduced by the dependence of ligand binding upon subunit interaction, and can be considered energetically identical with a system in which only 1 mol of each ligand is bound per mol of protein.

Materials and Methods

Chemicals. Chicken heart lactate dehydrogenase (P-L Biochemicals) was purchased as an ammonium sulfate suspension. Except for binding studies the enzyme was stored and handled at 4°C. Concentrations of enzyme solutions were determined from the adsorption at 280 nm, based on an absorption coefficient of $2.05 \times 10^5\ M^{-1}\ cm^{-1}$ (Pesce et al., 1964) and activity was determined by the assay developed by the same authors. Activities of the various lots of enzyme used during these experiments varied between 250 and 370 units per mg of protein, depending upon the age and lot of the preparation, but all lots had identical binding properties. Acrylamide gel electrophoresis of the enzyme on 5% T-2½% C gels (notation of Chrambach and Rodbard,

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[1] Abbreviations used are: NADH, reduced nicotinamide adenine dinucleotide; LDH, lactate dehydrogenase.

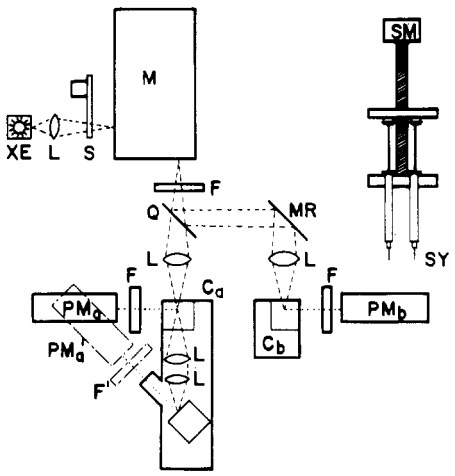

FIGURE 1: Plan view of fluorescence titrator: XE, 150-W xenon arc lamp; L, quartz lens; S, motor-driven shutter; M, Bausch & Lomb 0.5-m monochromator; F, filter holder; Q, quartz plate beam splitter; MR, mirror; C_a and C_b, water-jacketed sample compartments; PM_a and PM_b, photomultiplier tubes; PM_a' F', alternative geometry for front face fluorometry; SY, Hamilton gas-tight syringes; SM, Slo-Syn stepping motor and lead screw.

1971) showed the enzyme to have only one isozyme as detected by Coomassie Brilliant Blue 250 staining (Vesterberg, 1971) of heavily overloaded gels.

Lactate dehydrogenase solutions for binding studies were prepared by extensive dialysis of the ammonium sulfate suspension against 0.1 *M* pH 7.5 potassium phosphate buffer. The dialysate was then centrifuged at 29,000*g* for 20 min to remove insoluble material. Although this step did not significantly reduce the protein concentration as determined by the absorption at 280 nm, fluorescence in the 400–450-nm region was greatly reduced. It was noted that contaminants which oxidized small amounts of NADH in the presence of enzyme could be easily introduced by contact of the solutions involved with the skin. Accordingly special precautions (gloves) were taken in the handling of the enzyme.

NADH was purchased from Sigma Chemical Company, both in preweighed vials and in bulk. Binding and activity characteristics of both types were identical. [^{14}C]Oxalate was purchased from New England Nuclear and had a specific activity of 0.031 Ci/g. Quinine bisulfate was recrystallized from ethanol–water (Knopp and Weber, 1969). Other chemicals used were reagent quality and were used without further purification.

Apparatus. Fluorescence emission spectra were recorded on the scanning photon-counting fluorometer described by Jameson et al. (1974). Fluorescence lifetimes were measured on the phase-modulation cross-correlation fluorometer described by Spencer and Weber (1969). All lifetimes were measured with a polarizer in the exciting light beam oriented at 35°C from the vertical to cancel the effects of Brownian rotation on the lifetime (Spencer and Weber, 1970). All lifetime measurements were made at a modulation frequency of 14.2 MHz. For enzyme assays and uv absorption measurements, either a Cary 15 or a Zeiss PMQ II was used. Water-jacketed cuvet holders were used on all instruments except the Zeiss spectrophotometer.

Fluorescence Titrator. The fluorescence titration instrument shown in Figure 1 is a modified version of the instrument originally described by Anderson and Weber (1965). A motor-driven shutter (S) blocks the light path before the monochromator to reduce photolysis of the sample between measurements. A filter holder (F) in the exciting light beam is placed before the quartz plate beam splitter (Q) to facilitate removal of scattered or stray light and higher orders passed by the grating monochromator (M). The water-jacketed cuvet holder (C_a) holds 2 cm × 2 cm cuvets in either a right angle or front face optical configuration, while cuvet holder C_b holds a 2 cm × 2 cm cuvet in only the right angle configuration. Smaller cuvets are accommodated with brass adaptors.

Up to three Hamilton gas-tight syringes can be used to make simultaneous additions to the sample compartment(s) through polyethylene tubing. Precise additions are made by driving the syringes with a lead screw controlled by a stepping motor (SM). Although a reference solution can be placed in compartment C_b, the more usual content was a magnesium oxide coated scatterer, enabling the exciting light to be monitored.

After addition of titrant, the resulting solution in a cuvet compartment is mixed by the slow vertical motion of a perforated Teflon plate. This type of motion appears both to thoroughly mix the contents of the cuvet more rapidly than rotary stirring and to denature dilute solutions of the enzyme much less. The parts of the stirrer in contact with solution are made of Teflon and stainless steel to minimize contamination of the solution under study.

The desired regions of fluorescence emission are isolated by appropriate glass or interference filters and detected by EMI 6256B phototubes. The outputs of the phototubes are amplified by high gain current-to-voltage amplifiers utilizing FET operational amplifiers and an active filter stage. The resulting signals are ratioed and converted to a digital reading on a ratio digital voltmeter; 20–50 such readings are then averaged.

Procedures. Enzyme solutions are prepared in 0.1 *M* pH 7.5 potassium phosphate buffer. The enzyme concentration is kept approximately equal to the dissociation constant of the fluorescent ligand under study; 0.5–15-μl increments of a solution of the fluorescent ligand of concentration approximately 1000 times the ligand dissociation constant are added to 10.0 ml of enzyme solution in the titration instrument and the fluorescence (F) is measured. Next, the same increments of the ligand solution are added to 10.0 ml of a solution of buffer and any other components present in the previous solution except enzyme. The fluorescence (F_0) of the resulting solution is the background fluorescence. Finally, an acid solution of quinine bisulfate of the same absorption at the exciting wavelength as the ligand is added to 0.1 *N* sulfuric acid in the same increments used for the additions of ligands to protein, and the fluorescence (F_{qs}) is measured. As is derived in the Appendix, the average number of ligands bound per enzyme molecule, $\bar{n}$, and the concentration of free ligand, [X], at any point in a titration are given by

$$\bar{n} = \frac{F - F_0}{F_{qs}/B - F_0} \frac{[X]_t}{[P]_t} \quad (2)$$

$$[X] = [X]_t - \bar{n}[P]_t \quad (3)$$

where $[X]_t$ and $[P]_t$ are the total ligand and protein concentrations, respectively. B is a constant equal to the ratio of the relative signals produced by equal OD's of completely bound ligand and quinine bisulfate.

The binding of oxalate to lactate dehydrogenase in the presence of NADH is measured by adding increments of oxalate to a solution in potassium phosphate buffer of lac-

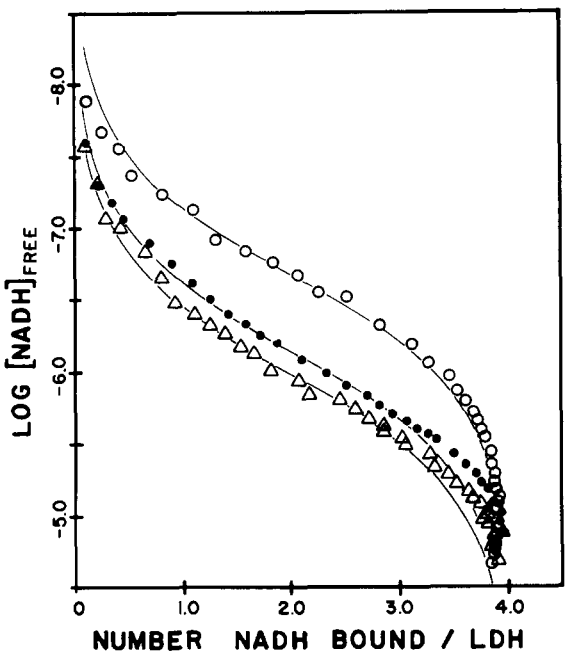

FIGURE 2: Effect of oxalate on the binding of NADH by lactate dehydrogenase at 20°. (Δ) Binding of NADH in the absence of oxalate; (●) binding of NADH in the presence of 1.0×10^{-4} *M* oxalate; (○) binding of NADH in the presence of 3.5×10^{-2} *M* oxalate. Lactate dehydrogenase concentration was 5.0×10^{-7} *M* in 0.1 *M* pH 7.5 potassium phosphate buffer. Solid curves are theoretical plots for dissociation constants of 1.7×10^{-6} *M* (*K*(NADH)), 7.4×10^{-7} *M*, and 2.0×10^{-7} *M* (*K*(NADH-oxalate)), respectively.

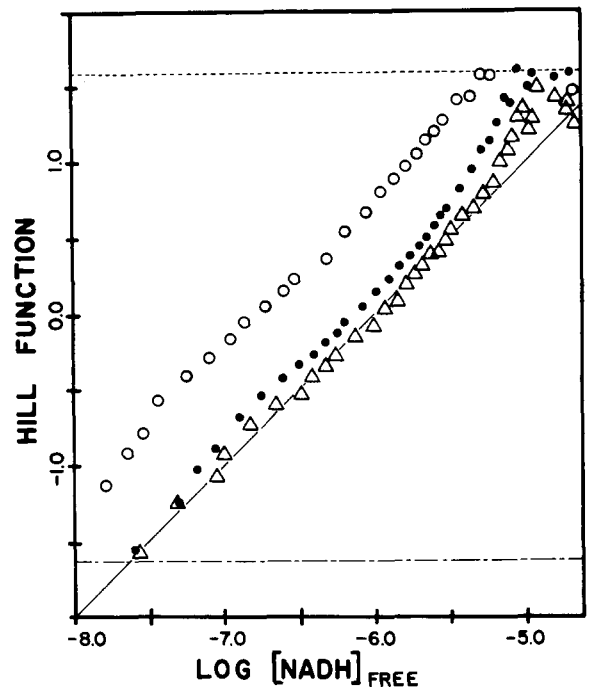

FIGURE 3: Hill plot of the binding of NADH by lactate dehydrogenase at 20°. (Δ) Binding of NADH in the absence of oxalate; (●) binding of NADH in the presence of 1.0×10^{-4} *M* oxalate; (○) binding of NADH in the presence of 3.5×10^{-2} *M* oxalate. Lactate dehydrogenase concentration was 5.0×10^{-7} *M* in 0.1 *M* pH 7.5 potassium phosphate buffer. Solid line corresponds to a Hill coefficient of 1 and a dissociation constant of 1.7×10^{-6} *M*. (- - -) 10% saturation of the binding sites with oxalate yields this Hill function; (- - -) 90% saturation of the binding sites with oxalate yields this Hill function.

tate dehydrogenase and NADH of sufficient concentration to saturate all of the NADH binding sites. Either the relative fluorescence or the fluorescence lifetime is then measured after each increment. Identical increments of buffer solution are added to a fresh solution of lactate dehydrogenase and NADH to obtain lackground readings and the fractional saturation of oxalate sites is then computed as derived in the Appendix.

NADH fluorescence is measured by excitation at the 328-nm isosbestic point for absorption by free and bound NADH. Relative fluorescence is measured as the emitted light passed at 432 nm by an interference filter with 10-nm band bass, rather than as the entire emitted spectrum, in order to increase the difference between the fluorescence intensities of bound and free NADH. Fluorescence lifetimes are measured as the average over the emission band passed by a 3-73 Corning glass filter.

Equilibrium dialysis titrations are carried out in dialysis cells of 0.5 ml capacity on each side of a dialysis membrane; 400 μl of a concentrated enzyme solution is placed on one side of the membrane and 400 μl of radioactive ligand solution on the other side. The cells are packed in ice and allowed to equilibrate for 5 days. Shorter time periods were tried but equilibrium was not always reached. Dual 100-μl samples are then removed from each chamber for liquid scintillation counting. In addition, 100 μl is also removed from the enzyme side of the membrane to assay for activity. Sampling precision is 0.5% and counting precision is 0.3%.

Assays for lactate dehydrogenase activity were performed on enzyme solutions before and after binding studies, as well as on control enzyme solutions which were allowed to stand at the same temperatures for the same times as those encountered in the binding studies. In all cases there was about 10% loss in activity during the binding studies, but this was no more than the activity lost by the controls over a similar period of time. Thus, the procedures used to study binding did not introduce additional changes in enzymatic activity.

Results

Binding of NADH by Lactate Dehydrogenase. Lactate dehydrogenase was confirmed to have four equivalent sites for NADH binding per tetramer unit of enzyme as measured from the intercept of the asymptotes of a plot of fluorescence of NADH vs. ratio of total concentration of NADH in solution to total concentration of lactate dehydrogenase. By the fluorescence titration procedure, the dissociation constant for NADH, *K*(NADH), as determined by binding studies on 5×10^{-7} *M* lactate dehydrogenase at 20°C was found to be $1.7 \pm 0.2 \times 10^{-6}$ *M*. A Bjerrum plot of the data had a normal span of 1.9 log units of NADH concentration (Figure 2), and a Hill plot of the data had a constant slope of 1 (Figure 3), indicating that binding of NADH was by noninteracting sites. In the presence of low (10^{-6} *M*) concentrations of oxalate there were no perceptable changes in the NADH binding from that observed in the absence of oxalate. At higher concentrations of oxalate (10^{-3} *M*) the binding of NADH was stronger than in the absence of oxalate as shown by a shift of the Bjerrum plot to lower NADH concentrations, but no cooperativity in the binding of NADH was observed. At high oxalate concentrations (0.1 *M*) the Bjerrum plot was shifted to the limiting value of $2.0 \pm 0.3 \times 10^{-7}$ *M*, corresponding to a shift of 0.94 log unit in NADH concentration. At this limiting value of the dissociation constant for NADH in the presence of oxalate, *K*(NADH/oxalate), there was still no coo-

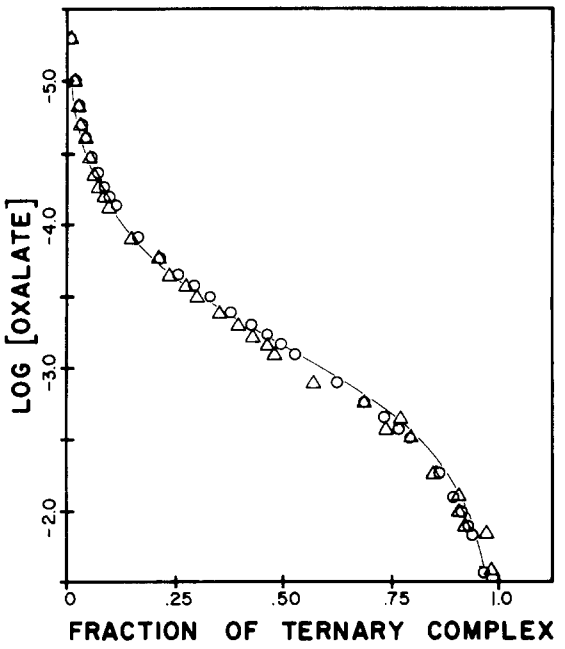

FIGURE 4: Binding of oxalate by NADH–lactate dehydrogenase at 20°. (○) Binding of oxalate calculated from change in the fluorescence yield of NADH at 432 nm; (△) binding of oxalate calculated from the change in fluorescence lifetime of NADH. NADH concentration was 3.0×10^{-5} *M*; LDH concentration was 3.0×10^{-6} *M*. Solid curve is a theoretical plot for a dissociation constant of 6.8×10^{-4} *M* (*K*(oxalate-NADH)).

perativity in the binding of NADH. The observed shift in dissociation constant for NADH corresponded to an interaction energy ΔF_{xy} (Weber, 1972) between oxalate and NADH of -1.3 ± 0.2 kcal/mol.

Binding of Oxalate by Lactate Dehydrogenase. Binding of oxalate to lactate dehydrogenase containing bound NADH was detected by the increase in quantum yield at 432 nm of the bound NADH. The NADH concentration was maintained at 3×10^{-5} *M* so that the ratio of bound to free NADH was constant throughout the titration and would not contribute any changes to the fluorescence quantum yield at 432 nm. Assuming that the increases in fluorescence yield was due to the formation of ternary complex only, the measured fluorescence change was proportional to the saturation of oxalate sites interacting with NADH. Alternatively, the fluorescence lifetime was measured over the entire emission band. Correction for the fact that the fluorescence lifetime is a nonlinear function of ternary complex formed (see Appendix) allowed the calculation of the Bjerrum curve shown in Figure 4. The Bjerrum plot of the fraction of ternary complex formed as calculated from fluorescence lifetimes is superimposable on the Bjerrum plot of the fraction of ternary complex formed as calculated from the change in fluorescence yield. In addition, the method of calculation from fluorescence lifetimes independently yields calculated lifetimes of 0.37 nsec for NADH free in solution and 0.79 nsec for NADH bound to lactate dehydrogenase in the absence of oxalate. The respective experimental values are 0.4 ± 0.03 and 0.8 ± 0.03 nsec. The dissociation constant for oxalate in the presence of NADH, *K*(oxalate/NADH), is found to be $6.8 \pm 0.4 \times 10^{-4}$ *M* at 20°C.

Binding of oxalate by lactate dehydrogenase in the absence of NADH was determined by equilibrium dialysis with [^{14}C]oxalate at 0°C. At an enzyme concentration of 4.1×10^{-5} *M* and oxalate concentrations between 3×10^{-4} and 3×10^{-3} *M*, the dissociation constant for oxalate, *K*(oxalate), was $4.3 \pm 1.5 \times 10^{-3}$ *M*. The observed ratios of [^{14}C]oxalate from enzyme side to buffer side of the dialysis membrane varied from 1.02 to 1.06, well above the range of experimental error (±0.5%). At the measured dissociation constant, the ligand concentrations are such that the binding sites for oxalate on the protein are between 10 and 30% saturated, and thus within a region of reasonable accuracy as derived by Deranleau (1969). The observed change in oxalate binding to lactate dehydrogenase when NADH is added from the binding in the absence of NADH corresponds to a change of 0.8 ± 0.14 log unit in the oxalate dissociation constant, or an interaction energy of -1.1 ± 0.3 kcal/mol between oxalate and NADH.

Conclusions

These are certainly straightforward:

$$\Delta F^\circ(\text{NADH/oxalate}) - \Delta F^\circ(\text{NADH}) = -1.3 \mp 0.2 \text{ kcal}$$

$$\Delta F^\circ(\text{Oxalate/NADH}) - \Delta F^\circ(\text{Oxalate}) = -1.1 \mp 0.3 \text{ kcal}$$

The two differences may be considered identical within experimental error and thus the reciprocity of the effects is quantitatively proven. One small point concerns that fact that three of the four constants were determined at 20°C and the fourth, ΔF°(oxalate), had to be determined at a temperature close to 0°C because of the long time necessary to achieve equilibrium in the dialysis experiments. To correct ΔF°(oxalate) to the value at the higher temperature requires knowledge of ΔH°(oxalate) the standard enthalpy change. If $\Delta F^\circ \simeq \Delta H^\circ$, that is if the free energy is entirely of enthalpic origin, ΔF°(oxalate/NADH) − ΔF°(oxalate) = −1.3 kcal, while if $\Delta H^\circ = 0$ it remains unchanged at −1.1 kcal. In either case the reciprocity is within experimental errors.

The thermodynamic requirement for reciprocity follows necessarily if the ternary complex formed by the protein and the two ligands is the same, irrespective of the order of addition of the reactants, but in systems of the complexity of protein molecules this prerequisite cannot be taken absolutely for granted. However, in view of the satisfactory quantitative demonstration provided here, and the many qualitative observations recorded, we propose that, unless rigorous proof to the contrary is presented, reciprocity must be considered to hold accurately, and all structural and dynamic schemes that violate it be discarded. The literature on binding by proteins often shows proposals that violate the reciprocity principle, and many more cases in which obvious conclusions are missed by failure to apply it.

Appendix

Calculation of both the average number of ligands bound per protein molecule and the concentration of free ligand from the fluorescence intensity is by a modification of the procedure of Daniel and Weber (1966). Any fluorescent species in solution emits a fluorescence intensity, *F*, which can be measured and which is proportional to the intensity of the incident light, I_0, the absorption of the emitting species, *A*, the quantum yield of the emitting species, *Q*, and instrumental efficiency, *C*

$$F = I_0AQC \qquad \text{(A.1)}$$

If different species present are excited at a common isosbestic point, then the absorption of each species is proportional to its concentration. Monitoring the exciting light with a

reference detector allows the intensity of exciting light to be treated as a constant. For a given set of instrumental conditions the observed fluorescence can thus be treated as a function of only the quantum yield and the concentration of each fluorescent species.

If Q_0 is the quantum yield of a fluorescent species, X, present in aqueous solution at a total concentration $[X]_t$, $Q_0 + \Delta Q$ is the quantum yield of species X when it is bound to a protein, F_0 is the fluorescence observed when no X is bound, and F is the fluorescence observed when some fraction α of the species X is bound to the protein, then

$$F_0 = Q_0[X]_t D \tag{A.2}$$

where D is a proportionality constant for instrumental settings and exciting light intensity.

$$F = (\alpha(Q_0 + \Delta Q) + (1 - \alpha)\, Q_0)[X]_t D \tag{A.3}$$

$$F = (Q_0 + \alpha\Delta Q)[X]_t D$$

When all of species X is bound to protein, the observed fluorescence, F_s, is

$$F_s = (Q_0 + \Delta Q)[X]_t D \tag{A.4}$$

To achieve this condition experimentally, the protein must be present in large excess over the ligand concentration. However, a typical binding experiment covers at least two orders of magnitude in ligand concentration, usually ending at a ligand concentration on the order of the highest possible protein concentration, making it impossible to achieve conditions of stoichiometric binding over the entire range of ligand concentrations observed. If eq A.4 holds for a limited range of ligand concentrations, however, F_s can be compared in this stoichiometric region to the fluorescence, F_{qs}, of a solution of quinine bisulfate of equal absorption in a solution of 0.1 N sulfuric acid

$$F_{qs} = F_s B \tag{A.5}$$

where B is an experimentally determined constant. After verification of this relationship in the measurably stoichiometric region of ligand binding, it is assumed to hold true for all stoichiometric binding.

Combining eq A.2–A.5

$$\frac{F - F_0}{F_{qs}/B - F_0} = \frac{(\alpha\Delta Q + Q_0 - Q_0)[X]_t D}{(\Delta Q + Q_0 - Q_0)[X]_t D} = \alpha \tag{A.6}$$

If $[P]_t$ is the total concentration of protein present, the average number of ligands bound per protein, $\bar{n}$, is then

$$\bar{n} = \frac{\alpha[X]_t}{[P]_t} = \frac{F - F_0}{F_{qs}/B - F_0}\frac{[X]_t}{[P]_t} \tag{A.7}$$

where all of the unknowns can be experimentally determined. Similarly, the concentration of free ligand, [X], is

$$[X] = [X]_t - \bar{n}[P]_t \tag{A.8}$$

Since all measurements are made under the same instrumental conditions, even optical factors which are included in the constant D are cancelled out without the need for involved inner filter corrections.

Calculation of the Saturation of Oxalate Binding Sites from the Measured Change in the Fluorescence Lifetime of Bound NADH. The fluorescence lifetime measured by the cross-correlation phase fluorometry method of Spencer and Weber (1969) is a weighted average of the lifetimes of all fluorescent species present. The average value of the tangent of the phase delay angle measured by this method, $\overline{\tan}\ \overline{\phi}$, is a function of the amplitudes of the modulated photocurrents, a_i, from each emitting species and the phase delay angle, ϕ_i, due to that species alone:

$$\overline{\tan\phi} = \frac{\sum_i a_i \sin\phi_i}{\sum_i a_i \cos\phi_i} \tag{A.9}$$

The amplitude of the modulated photocurrent is equal to $f_i Q_i \cos\phi_i$, where f_i is the fraction of the total excited population present as species i and Q_i is the relative quantum yield of that species. Therefore

$$\overline{\tan\phi} = \frac{\sum_i f_i Q_i \tan\phi_i \cos^2\phi_i}{\sum_i f_i Q_i \cos^2\phi_i} \tag{A.10}$$

Setting $\omega\bar{\tau} = \overline{\tan\phi}$ where ω is the modulation frequency, the average measured lifetime, $\bar{\tau}$, is related to the lifetimes of the individual species, τ_i, by

$$\bar{\tau} = \frac{\sum_i [\tau_i/(1 + \omega^2\tau_i^2)]f_i Q_i}{\sum_i [1/(1 + \omega^2\tau_i^2)]f_i Q_i} \tag{A.11}$$

In the NADH–oxalate–lactate dehydrogenase ternary system there are three fluorescent species: NADH free in aqueous solution, NADH bound to a lactate dehydrogenase subunit with no interaction with oxalate, and NADH bound to a lactate dehydrogenase subunit and interacting with oxalate, states 1, 2, and 3, respectively. If the concentration of NADH present is in large excess over the protein concentration, then f_1 is constant. (From the measured NADH dissociation constants, at an NADH concentration of 3×10^{-5} M and a lactate dehydrogenase concentration of 3×10^{-6} M, f_1 is 0.61 in the presence or absence of oxalate.) For convenience, Q_3 is assumed to be 1. The experimentally observed ratio of Q_2/Q_1, $C = 2.15 \pm 0.05$ when integrated over the emission spectra, is in agreement with the value found by Winer and Schwert (1959). If all quenching of fluorescence is dynamic, a circumstance that seems to apply to all cases of NADH derivatives (Scott et al., 1970, Figure 5), then the change in observed fluorescence is proportional to the amount of ternary complex formed.

For the present system, if i, j = 1, 2, 3

$$\tau_i/\tau_j = Q_i/Q_j \tag{A.12}$$

In the absence of oxalate the measured lifetime is the minimum measured, τ_{min}, and f_3 is 0. From eq A.12 and the observed ratio of Q_2/Q_1, C, substitution into eq A.11 and rearrangement yields

$$\bar{\tau} = \bar{\tau}_{min} = \frac{\tau_2}{C}\frac{0.61(1 + \omega^2\tau_1^2) + 0.39C^2(1 + \omega^2\tau_1^2/C^2)}{0.61(1 + \omega^2\tau_2^2) + 0.39C(1 + \omega^2\tau_2^2/C^2)} \tag{A.13}$$

This equation can be reduced to a standard form cubic equation in τ_2 and solved for the real root. τ_1 is then $\tau_2/2.15$.

When oxalate is present in large excess such that all of its sites on the protein are occupied, f_2 is 0 and the observed lifetime is a maximum, τ_{max}. By substituting τ_3/τ_1 for Q_3/Q_1, eq A.11 can be rearranged to

$$\tau_{max} = \frac{(0.39\tau_3^2/\tau_1) + [0.61\tau_2(1 + \omega^2\tau_3^2)/(1 + \omega^2\tau_1^2)]}{(0.39\tau_3/\tau_1 + [0.61(1 + \omega^2\tau_3^2)/(1 + \omega^2\tau_1^2)]} \tag{A.14}$$

Which is a quadratic equation in τ_3. Since τ_1 was previously determined, τ_3 is the positive root of this equation, and Q_2 and Q_1 can be found.

Let

$$A = \frac{Q_1}{1 + \omega^2\tau_1^2}$$
$$B = \frac{Q_2}{1 + \omega^2\tau_2^2}$$
$$C = \frac{Q_3}{1 + \omega^2\tau_3^2} \qquad (A.15)$$
$$f_2 = 0.39 - f_3$$

substitution into eq A.11 and rearrangement yields

$$f_3 = \frac{0.61A(\bar{\tau} - \tau_1) + 0.39(\bar{\tau} - \tau_2)B}{C(\tau_3 - \bar{\tau}) + B(\bar{\tau} - \tau_2)} \qquad (A.16)$$

The degree of saturation of oxalate binding sites, S, is then

$$S = f_3/0.39 \qquad (A.17)$$

References

Anderson, S. R., and Weber, G. (1965), *Biochemistry 4,* 1948–1957.

Antonini, E., Wyman, J., Brunori, M., Bucci, E., Fronticelli, C., and Rossi-Fanelli, A. (1963), *J. Biol. Chem. 238,* 2950–2957.

Benesch, R., Benesch, R. E., and Yu, I. C. (1968), *Proc. Natl. Acad. Sci. U.S.A. 59,* 526–532.

Chrambach, A., and Rodbard, D. (1971), *Science 172,* 440–451.

Daniel, E., and Weber, G. (1966), *Biochemistry 5,* 1893–1900.

Deranleau, D. A. (1969), *J. Am. Chem. Soc. 91,* 4044–4049.

Jameson, D. M., Spencer, R. D., and Weber, G. (1974), *Fed. Proc., Fed. Am. Soc. Exp. Biol. 33,* 1321

Knopp, J. A., and Weber, G. (1969), *J. Biol. Chem. 244,* 6309–6315.

Nowak, T., and Mildvan, A. S. (1972), *Biochemistry 11,* 2819–2828.

Pesce, A., McKay, R. H., Stolzenbach, F., Cahr, R. D., and Kaplan, N. O. (1964), *J. Biol. Chem. 239,* 1753–1761.

Scott, T. G., Spencer, R. D., Leonard, N. J., and Weber, G. (1970), *J. Am. Chem. Soc. 92,* 687–695.

Spencer, R. D., and Weber, G. (1969), *Ann. N.Y. Acad. Sci. 158,* 361–376.

Spencer, R. D., and Weber, G. (1970), *J. Chem. Phys. 52,* 1654–1663.

Vesterberg, O. (1971), *Biochim. Biophys. Acta 243,* 345–348.

Weber, G. (1972), *Biochemistry 11,* 864–878.

Weber, G. (1975), *Adv. Protein Chem. 29,* 1–83.

Winer, A. D., and Schwert, G. W. (1959), *J. Biol. Chem. 234,* 1155–1166.

Winer, A. D., Schwert, G. W., and Millar, D. B. S. (1959), *J. Biol. Chem. 234,* 1149–1154.

Wyman, J. (1964), *Adv. Protein Chem. 19,* 223–286.

Cooperativity of Binding of Anilinonaphthalenesulfonate to Serum Albumin Induced by a Second Ligand†

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ABSTRACT: When a ligand X is multiply bound to energetically identical, noninteracting sites of a protein, cooperative binding of this ligand can be induced by the presence of a second ligand Y. This effect should appear whenever multiple interactions exist between the bound X and Y ligands, and vanish when the concentration of Y is made sufficiently large to ensure Y saturation at all concentrations of X. These predictions have been verified for the binding of 8-anilino-1-naphthalenesulfonate to serum albumin, when Y, the effector ion, is 3,5-dihydroxybenzoate. In the presence of 2 mM dihydroxybenzoate, the Hill coefficient for anilinonaphthalenesulfonate binding rose steadily from 1 to 1.5 as the number of molecules of ligand bound increased from 1 to 3.3 per albumin molecule. The theory of interactions between isolated ligands, applied in the previous paper (D. A. Kolb and G. Weber (1975), *Biochemistry,* preceding paper in this issue), is extended to cases of multiple interactions, and applied here to show that the experimental results are tolerably well reproduced for a model in which four anilinonaphthalenesulfonate molecules are homogeneously coupled to four molecules of dihydroxybenzoate by free energies of 3.0 and 3.5 thermal units.

Cooperative ligand binding by proteins is classically exemplified by the equilibria of several ligands with hemoglobin (Wyman, 1964) and has more recently been observed in other oligomeric proteins (Changeux et al., 1968; Er-el et al., 1972). In many more cases the dependence of catalytic reaction velocity upon substrate concentration has been considered to reflect cooperative substrate binding (Kirschner, 1968). Monod et al. (1965) explained these cooperative effects as arising out of the requirement for symmetry conservation that they postulated as operating in oligomeric protein aggregates. On the other hand, Weber (1972) has shown that cooperative binding of a multiply bound ligand X arises inevitably as a result of positive or negative *multiple* interactions between two or more molecules of X on one

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