Quantitative Analysis of Protein:Immobilized Dye Interaction*

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The interaction of rabbit muscle lactate dehydrogenase with reactive blue 2 immobilized on Sepharose CL-6B was quantitatively evaluated by analytical procedures using both frontal and zonal chromatography and also static equilibrium methodology. All three analytical procedures gave a common result, namely (i) that each enzyme molecule is retained by a single immobilized dye molecule; (ii) that the immobilized dye binds to the protein at an NADH binding site with an affinity identical to that of the free dye; and (iii) that less than 2% of the immobilized dye is accessible to the enzyme. The very small fractional accessibility is not due to steric exclusion but likely is a result of adsorption of the immobilized dye to the matrix surface.

Mobile textile dyes have been found to bind with relatively high affinity to a wide range of proteins (1). Such interactions have been exploited to great advantage in the purification of proteins using dyes immobilized to porous matrices (2). Since textile dyes are designed to adsorb to surfaces, many investigators view these dyes as capable of binding to a multitude of surfaces on a given protein. Because immobilized dye columns used in the purification of proteins frequently contain high concentrations of dye to increase protein capacity, the opportunity for a multiplicity of immobilized dyeprotein interactions exists, a situation termed multivalency in the jargon of affinity chromatography.

We chose to investigate the nature of immobilized dye-protein interactions using the general quantitative procedures available for characterization of immobilized ligands. Reactive blue 2, color index 61211, was used as a model immobilized dye since it is commonly used in enzyme purification protocols. Lactate dehydrogenase was selected as a model protein because it contains four identical sites whose interaction with mobile reactive blue 2 has been well characterized (3). Thus, both homogeneous as well as heterogeneous interactions between the model protein and the immobilized dye are potentially feasible. Finally, we constructed an immobilized reactive blue 2 column presumed to have a random distribution of covalently attached dye having a concentration of 120 \( \mu M \). This concentration is in excess of the immobilized hapten, 50 \( \mu M \), demonstrated to generate bivalent homogeneous interactions with dimeric immunoglobulin (4).

EXPERIMENTAL PROCEDURES

Materials—Purified rabbit muscle lactate dehydrogenase was purchased from Sigma and dialyzed exhaustively against 100 mM phosphate buffer, pH 7.5, prior to use. The dialyzed enzyme has a specific catalytic activity of 360 units/mg and migrated as a single component when examined by polyacrylamide electrophoresis both in the presence and absence of sodium dodecyl sulfate. Purified reactive blue 2 was purchased from Polysciences and found to contain at least 99% blue dye by adsorption chromatography.

The triazine ring of the dye was covalently attached to Sepharose CL-6B as described by Heyns and De Moor (5). The dye-conjugated Sepharose was then washed using the protocol of Haff and Easterday (6) until no detectable color eluted. The quantity of dye retained by the washed Sepharose was then measured spectrophotometrically following acid hydrolysis as described by Chambers (7). The conjugated Sepharose CL-6B used throughout this study was 120 \( \mu M \) in reactive blue 2.

All chromatographic analyses were done at 23 °C using an immobilized dye column (0.8 X 2.3 cm bed size). All solutions introduced to the column were 100 mM in phosphate buffer, pH 7.5. The concentration of lactate dehydrogenase was measured in terms of its catalytic activity using the standard spectrophotometric assay procedure (8). All chromatographic fractions contained 0.11 ml. All concentrations are given as micromolar, and complexation constants are reported as dissociation constants having the units of micromolar concentration.

Frontal chromatographic analyses were done by continuous application of a series of solutions of lactate dehydrogenase in the presence and absence of NADH. Each solution which had a fixed concentration of lactate dehydrogenase and of NADH was applied to the column at a flow rate of 130 \( \mu l/\)min until the concentration of lactate dehydrogenase in the effluent fractions attained a constant value termed the plateau concentration. Results were analyzed using Equation 1 which in its general form was introduced by Nichol et al. (9) to describe a single interaction between an immobilized ligand and a mobile protein, a situation termed monovalent interaction.

\[
\frac{1}{V_i - V} = \frac{[\text{LDH}]}{V_i[DYE]} + \frac{1}{(V_i[DYE])} \left( \left( \frac{K_{\text{NADH}}}{(\text{NADH})} \right) \left( \frac{K_{\text{NADH}}}{(\text{DYE})} \right) \right)
\]

where LDH = lactate dehydrogenase. Each of the volume terms in this equation represents the elution volume containing 50% of the plateau concentration of lactate dehydrogenase or the calibration compounds. The terms \( V_i \) and \( V_0 \) represent the elution volumes of lactate dehydrogenase from the conjugated dye column and from an unconjugated Sepharose CL-6B column having the same dimensions, respectively. The term \( V_i \) represents the difference in the elution volumes of the calibration compounds, potassium chromate and blue dextran. The term \( [\text{DYE}] \) represents the concentration of accessible immobilized dye and the terms \( K_{\text{DYE}} \) and \( K_{\text{NADH}} \) represent the dissociation constants of the lactate dehydrogenase:NADH binary complexes, respectively. This notation is used throughout the text.

Zonal chromatographic analyses were done by application of 100-\( \mu l \) portions of a 1.2 \( \mu M \) solution of lactate dehydrogenase to the immobilized dye column which was equilibrated and developed with a fixed concentration of NADH at a flow rate of 40 \( \mu l/\)min. Elution volumes were obtained by triangulation of the elution profiles as suggested by Chaiken and Taylor (10). Results were analyzed using Equation 2 which was developed in its general form by Dunn and Chaiken (11) for monovalent interactions.

\[
\frac{1}{V_i - V} = \frac{K_{\text{DYE}}}{(\text{DYE})} + \frac{K_{\text{DYE}}}{(\text{NADH})} \left( \frac{1}{(V_i[DYE])} \right) \left( \frac{1}{(V_i[DYE])} \right)
\]

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Again, the terms $V_1$ and $V_2$ represent the elution volumes for lactate dehydrogenase from dye-Sepharose CL-6B and from Sepharose CL-6B columns of equivalent dimensions, respectively. The term $V_v$ represents the void volume of the dye-Sepharose column as measured using blue dextran.

Static as opposed to flow measurements were done using 140-μl portions of the conjugated Sepharose. Exactly 250 μl of an enzyme solution were added to each portion of the conjugated Sepharose and the mixture was allowed to equilibrate for 2 h with gentle stirring. An aliquot of the supernatant was then analyzed using Equation 3 which was developed in its general form for monovalent interaction by Graves and Wu (12).

\[
\frac{(V + V_2)([LDH])}{(V)([LDH])} = \frac{(V + V_2)(K_{DYE})}{(V_2)([DYE])} + \frac{(V + V_2)([LDH])}{(V_2)([DYE])}
\]

In this equation, $V$ represents the volume in which the enzyme of concentration $[LDH]_0$ was delivered to the conjugated Sepharose; $V_v$ is the volume of Sepharose accessible to lactate dehydrogenase; and $[LDH]$ is the concentration of enzyme in the Sepharose supernatant at equilibrium.

**RESULTS**

The concentrations of immobilized reactive blue 2 accessible to lactate dehydrogenase were determined by frontal analysis of flow measurements and by analysis of static measurements. Typical results obtained by frontal analysis are shown in Fig. 1A. The dependence of the volume required for emergence of the enzyme on the concentration of enzyme solution applied describes a linear relationship over the concentration range examined, as shown in Fig. 1B. The slope and intercept of the relationship shown in Fig. 1B indicate that 1.7 μM immobilized dye is accessible to the enzyme and that the immobilized dye-lactate dehydrogenase complex has a dissociation constant of 0.23 μM. Results obtained by analysis of static measurements are shown in Fig. 2. The linear display is consistent with the monovalent interaction between immobilized dye and enzyme. The slope and intercept of this relationship indicate that 2.3 μM immobilized reactive blue 2 is accessible to lactate dehydrogenase and that the binary complex has a dissociation constant of 0.17 μM.

The effect of the mobile ligand, NADH, on the interaction between immobilized reactive blue 2 and lactate dehydrogenase during flow was examined by both frontal and zonal analysis. Typical emergence profiles of enzyme observed by frontal analysis in the presence and absence of NADH are shown in Fig. 3A. The dependence of the enzyme emergence volume on the concentration of NADH describes a linear relationship as shown in Fig. 3B. Such linearity is consistent with a simple competition between immobilized reactive blue 2 and NADH for a common site on the enzyme and with a monovalent interaction between immobilized reactive blue 2 and lactate dehydrogenase. Using the slope of the linear relationship shown in Fig. 3B and the values of $[DYE]$ and $K_{DYE}$ found by analysis of Fig. 1B, we obtain the dissociation constant of 2.4 μM for the NADH:lactate dehydrogenase binary complex from Equation 1. Typical elution profiles observed for the enzyme by zonal chromatography in the presence and absence of NADH are shown in Fig. 4A. The dependence of the elution volume of enzyme on the concentration of NADH present describes a linear relationship as
shown in Fig. 4B. Such linearity indicates that NADH and immobilized reactive blue 2 compete for a common site on the enzyme. The slope and intercept of the linear relationship described in Fig. 4B allow us to calculate the 

$$K_{	ext{DYE}} (0.19 \mu M)$$

and 

$$K_{	ext{NADH}} (3.7 \mu M)$$

from Equation 2 with the value of [DYE] found by analysis of Fig. 1B.

The retention of reactive blue 2 by unconjugated Sepharose CL-6B is shown in Fig. 5. Retention appears to occur in two phases, an initial hyperbolic phase and a limiting linear phase. We suggest that the results shown in Fig. 5 reveal an initial adsorption of dye to the matrix surface followed by a stacking of further dye on the matrix adsorbed dye. Since these two processes would be concurrent and stacking is not saturable, Fig. 5 does not readily bind itself to analysis. Nonetheless, the initial values shown in Fig. 5 suggest that the Sepharose matrix has a high affinity for mobile free dye.

**DISCUSSION**

Values for the concentration of accessible immobilized reactive blue 2 and for the dissociation constants of the lactate dehydrogenase:immobilized reactive blue 2 and the lactate dehydrogenase:NADH complexes obtained from the analyses described above are compared in Table I. It should be noted that the values obtained from both static and flow procedures as well as by frontal and zonal analysis of flow procedures are virtually identical. Further, the dissociation constants of the immobilized reactive blue 2:lactate dehydrogenase complex fall within the range of values reported for the dissociation constant of mobile reactive blue 2:lactate dehydrogenase complexes as measured by spectral titration, by catalytic inhibition, and by equilibrium dialysis (3, 13). Similarly, the dissociation constant for the NADH:lactate dehydrogenase complex obtained from measurements using the immobilized dye are within a factor of two of values obtained by fluorescence titration measurements (13).

These quantitative measurements both confirm and extend qualitative observations of the interaction of immobilized reactive blue 2 with lactate dehydrogenase and quantitative measurements of the interaction of the mobile dye with the enzyme. First, the immobilized dye binds to the NADH binding site on the enzyme, and since we have shown that the immobilized dye:protein interaction is monovalent, this site must have the greatest affinity for the immobilized dye. Second, immobilization of reactive blue 2 neither enhances nor diminishes the affinity of the dye for the enzyme. This observation is compatible with the view that the interaction between reactive blue 2 and proteins principally involves the terminal anthraquinone ring. Accordingly, it is likely that the dianisophenyl ring and the triazine ring which intervene between the anthraquinone ring and the Sepharose matrix serve as benign spacer groups.

Most surprising to us was the recognition that more than 98% of the immobilized dye is inaccessible to lactate dehydrogenase. While a fraction of immobilized bioligands are generally not accessible to protein, the fractional inaccessibility of the immobilized dye seems particularly high. Since textile dyes are designed to adsorb strongly to surfaces, we suspect that the majority of the conjugated dye is adsorbed to the Sepharose surface and is unavailable for complexation with lactate dehydrogenase. Such suspicion is supported by the retention of free dye by Sepharose shown in Fig. 5. Since the local concentration of matrix for immobilized dye as opposed to free dye is much greater, the adsorption of the immobilized dye to the matrix is likely very strong. We are currently probing the characteristics of this adsorption using chromatographic analyses.

The accessibility of only 2.0 ± 0.3 μM of immobilized reactive blue 2 to lactate dehydrogenase likely accounts for the monovalent interaction observed in this study in that the probability of a retained lactate dehydrogenase molecule simultaneously contacting two molecules would be quite low. By contrast, Yon and Kyrpantzou (13) have recently reported that an immobilized reactive blue 2 column having 31 μM dye accessible to lactate dehydrogenase gives evidence of multivalency by frontal chromatographic analysis. However, 31 μM accessible dye may not represent a lower boundary for detection of multivalency since they generated their column by a 5-fold dilution of a highly conjugated dye-Sepharose with unconjugated Sepharose. Such a procedure will reduce the concentration of beads containing conjugated dye but will not reduce the concentration of dye within the beads. Since immobilized reactive blue 2:lactate dehydrogenase interactions can be either monovalent or multivalent we suggest a systematic study be undertaken to determine the point of departure using randomly conjugated beads and to investigate whether the multivalency is homogeneous involving just NADH sites, heterogeneous involving two or more kinds of sites, or both.

In pursuit of such studies, we would suggest frontal chromatographic analysis as the procedure of choice given an adequate supply of protein, since (i) the concentration of accessible immobilized dye is determined in the course of the measurements, (ii) the method is not limited by the off rate of the immobilized ligand:protein complex, and (iii) equations for at least bivalent homogeneous interactions are available.

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Immobilized Reactive Blue 2

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