Journal of Chromatography, 376 (1986) 149—155
Biomedical Applications
Elsevier Science Publishers B.V., Amsterdam — Printed in The Netherlands

CHROMBIO. 2966

ZONAL CHROMATOGRAPHIC ANALYSIS OF THE INTERACTION OF ALCOHOL DEHYDROGENASE WITH BLUE-SEPHAROSE

YIN CHANG LIU and EARLE STELLWAGEN*

Department of Biochemistry, University of Iowa, Iowa City, IA 52242 (U.S.A.)

SUMMARY

The interaction between horse liver alcohol dehydrogenase and Reactive blue 2 immobilized on Sepharose CL-6B was measured by zonal chromatography. Each protein molecule was retained by a single immobilized dye using a Blue-Sepharose column containing a total of 1.38 m*M* dye. However, the protein was predominately retained by two immobilized dye molecules using a darker Blue-Sepharose column containing a total of 11.6 m*M* dye. The dissociation constant measured for the alcohol dehydrogenase—immobilized dye complex on each column is identical to the inhibition constant for the alcohol dehydrogenase—free Reactive blue 2 complex: 4.5 ± 0.8 μM.

INTRODUCTION

We have previously [1] analyzed the interaction between tetrameric rabbit muscle lactate dehydrogenase and Sepharose CL-6B lightly substituted with Reactive blue 2 using data collected by static equilibrium, by frontal chromatographic and by zonal chromatographic procedures. Such analyses gave a common result, namely that each tetrameric protein is retained by a single immobilized dye and that the dissociation constant for this complex is identical with the protein—free dye complex. In this report we use zonal chromatography to investigate the interaction between dimeric horse liver alcohol dehydrogenase and a Sepharose CL-6B more extensively substituted with Reactive blue 2. We have chosen to use alcohol dehydrogenase for these measurements in order to be assured by crystallographic measurements [2] that the dye specifically complexes with a single site on each protomer of the protein, namely the nicotinamide-adenine dinucleotide (NAD) binding site.

EXPERIMENTAL

Horse liver alcohol dehydrogenase was purchased from Sigma (St. Louis,

0378-4347/86/$03.50

MO, U.S.A.). The concentrations of solutions of the protein were measured by absorbance spectroscopy using an extinction [3] at 280 nm of 36.4 mM^{-1} cm^{-1} or by enzymic assay using the procedure of Vallee and Hoch [4] and a specific activity of 1.80 U/mg. Reactive blue 2 (Cibacron blue F3GA) was purchased from Fluka (Hauppauge, NY, U.S.A.). Solutions of this dye preparation migrated as a single component when examined by thin-layer chromatography using three different solvent systems [5]. Sepharose CL-6B was purchased from Sigma. All procedures were carried out at 25°C.

The triazine ring of Reactive blue 2 was covalently attached to Sepharose CL-6B using the procedure of Heyns and De Moor [6]. The product was washed extensively using the protocol of Haff and Easterday [7] until the effluent was free of material having a visible absorbance. The total amount of Reactive blue 2 retained by the washed Sepharose was determined spectrophotometrically following acid hydrolysis as described by Chambers [8]. The concentration of total immobilized dye is expressed in terms of the volume of the swollen packed conjugated matrix.

Zonal chromatography was carried out using a 1.0-ml bed volume of dye-substituted Sepharose placed in a plastic microcolumn. The Blue-Sepharose was equilibrated with 100 mM phosphate buffer, pH 7.5, containing a desired concentration of NAD. A 100-μl aliquot of enzyme in the same solvent containing 10 U of enzymic activity was applied to the top of the column and the column was then irrigated with the equilibration solvent at a flow-rate of 3 ml/h. Effluent fractions each containing 0.46 ml were collected and the enzymic activity of each fraction was determined.

The concentration of immobilized dye accessible to the protein was measured by a static equilibrium procedure. Exactly 50 μl of a suspension of Blue-Sepharose were placed in each of a series of Eppendorf centrifuge tubes. Between 1 and 150 μl of a 377 μM solution of alcohol dehydrogenase were added to each tube and the contents mixed overnight. The supernatant was then removed by centrifugation and the precipitate washed with five 100-μl aliquots of 100 mM phosphate buffer, pH 7.5. After removal of the last wash aliquot following centrifugation, the Blue-Sepharose was eluted with 150 μl of 100 mM phosphate buffer, pH 7.5, containing 5 mM NAD. The concentration of enzyme recovered in the eluate following centrifugation was determined by catalytic assay. It was initially assumed that each retained enzyme was complexed with a single immobilized dye. In those situations in which bivalence occurs, the concentration of accessible dye was increased commensurate with the fractional multivalency. The concentration of accessible matrix is expressed in terms of the volume of the swollen packed conjugated matrix.

Zonal chromatographic results were analyzed using eqns. 1 and 2 which have been developed by Hethcote and Delisi [9] and are formally identical with those derived by Dunn and Chaiken [10] and by Eilat et al. [11].

$$V_e = V_0 + V_p\left(1 + \frac{K_I[D]}{1 + K_L[L]}\right) \quad (1)$$

$$V_e = V_0 + V_p\left(1 + \frac{2K_1[D]}{1 + K_L[L]} + \frac{K_1K_2[D]^2}{1 + K_L[L]^2}\right) \quad (2)$$

In these equations, V_e is the volume of the maximum ordinate in the elution profile, V_0 is the void volume of the column, V_p is the volume of the column penetrated by the protein, K_1 and K_2 are association constants for the protein with immobilized ligand, [D] is the concentration of immobilized ligand accessible to the protein, K_L is the association constant for the protein with mobile ligand, and [L] is the concentration of mobile ligand. Eqn. 1 describes a monovalent interaction and eqn. 2 a divalent interaction. The void volume was determined using Blue dextran and was found to be 1.49 ml and the penetration volume of alcohol dehydrogenase was found to be 0.33 ml. Previous measurements [12] of the kinetics of interaction of Blue-Sepharose with lactate dehydrogenase have indicated that mass transfer within the column and not complexation is the rate-limiting step using the protocols employed here.

RESULTS

The dissociation constant for the enzyme—free dye complex was determined by catalytic measurements. Results of such measurements are shown in Fig. 1 in the form of a double reciprocal plot. The entire data set was subjected to non-linear least-squares analysis using various modes of inhibition. The best fit was obtained for competitive inhibition between free dye and NAD for the same form of the enzyme. The non-linear least-squares best fit for a competitive inhibition model gave an inhibition constant for the enzyme—free dye complex of 4.5 μM with a standard error of 0.8 μM. We take this value to represent the dissociation constant of the enzyme—free dye complex.

Two Blue-Sepharose columns were used in this study. One column contained

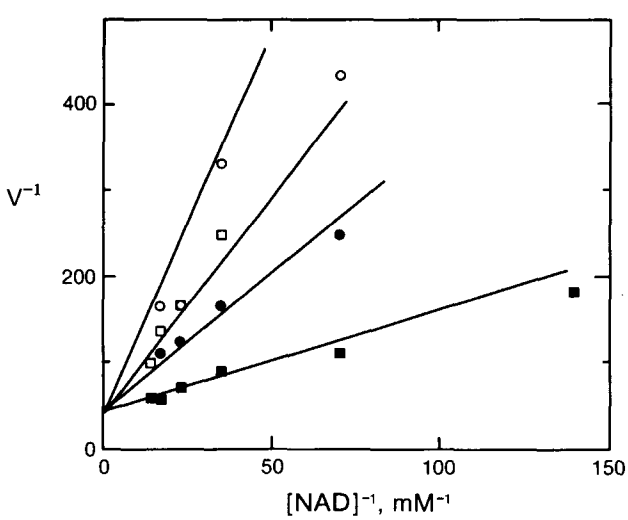

Fig. 1. Measurements of the catalytic rate of alcohol dehydrogenase in the presence of variable fixed concentrations of Reactive blue 2. All solutions contained 100 mM phosphate buffer, pH 7.5, and no dye (▪); 7.5 μM dye (•); 15 μM dye (□); or 30 μM dye (○).

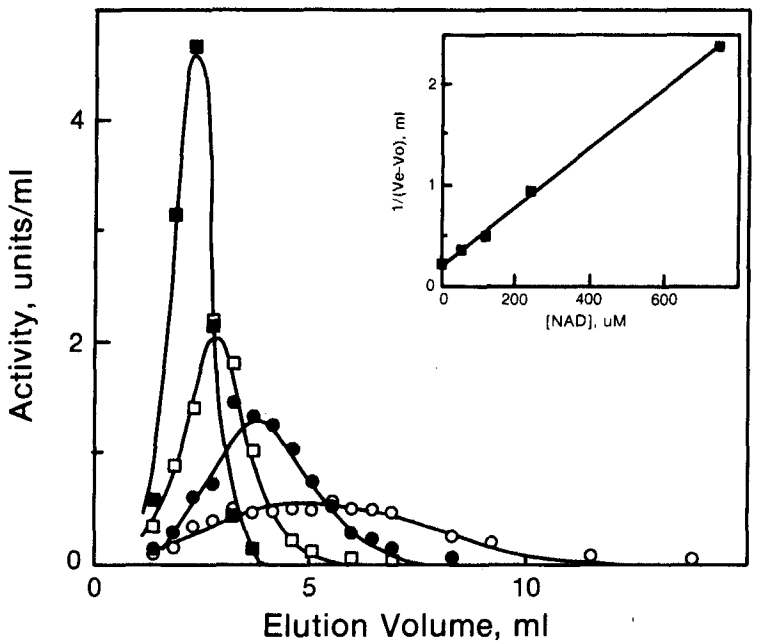

Fig. 2. Zonal chromatography of alcohol dehydrogenase using Blue-Sepharose containing 1.38 m*M* immobilized dye. The equilibration solvents all contained 100 m*M* phosphate buffer, pH 7.5, and 55 μM NAD (○); 122 μM NAD (●); 242 μM NAD (□); or 746 μM NAD (■). The inset represents an analysis of the results shown in the main portion of the figure in which V_e is the maximal ordinate of the elution profile for a given concentration of NAD and V_0 is the exclusion volume.

1.38 m*M* total immobilized dye of which at least 0.05 m*M* is accessible to alcohol dehydrogenase. The other column contained the maximal total immobilized dye which we could attain, 11.6 m*M*, of which a minimum of 0.116 m*M* is accessible to the enzyme. Elution profiles observed following zonal application of enzyme to the immobilized-dye column containing 1.38 m*M* total dye are shown in Fig. 2. It can be seen that the value for the maximum ordinate, V_e, of the elution profiles is inversely dependent upon the concentration of the mobile competitive ligand, NAD, in the equilibration solvent. This dependence describes a linear semireciprocal relationship shown in the inset to Fig. 2. Such linearity suggests [10] that each dimeric enzyme is retained by a single immobilized dye, i.e. the interaction is monovalent, and that the immobilized dye and NAD compete for a common site on the enzyme. Analysis of the dependence of V_e on the concentration of NAD in the elution solvent using eqn. 1 indicates that the monovalent enzyme–immobilized dye complex has a dissociation constant of 5.3 μM and that the enzyme–NAD complex has a dissociation constant of 66 μM. This latter value compares favorably with a dissociation constant of 110 μM obtained by spectrophotometric measurements at pH 7.0 [13].

Elution profiles observed following zonal application of enzyme to the immobilized-dye column containing 11.6 m*M* total immobilized dye are shown in Fig. 3. Since micromolar concentrations of NAD efficiently elute the enzyme, we assume that NAD and the immobilized dye compete for a common

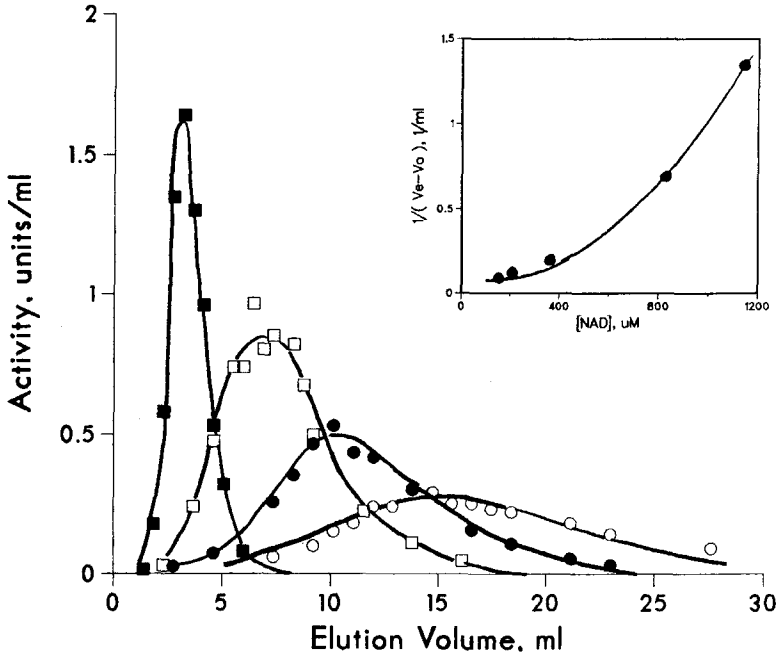

Fig. 3. Zonal chromatography of alcohol dehydrogenase using Blue-Sepharose containing 11.6 m*M* immobilized dye. All equilibration solvents contained 100 m*M* phosphate buffer, pH 7.5, and 158 μM NAD (○); 204 μM NAD (●); 367 μM NAD (□); or 835 μM NAD (■). The inset represents an analysis of the results shown in the main portion of the figure in which V_e is the maximal ordinate of the elution profile for a given concentration of NAD and V_0 is the exclusion volume.

site on the enzyme. The dependence of V_e on the concentration of NAD describes a curvilinear semireciprocal relationship as shown in the inset to Fig. 3. Such curvilinearity suggests that for some of the retained enzyme molecules both NAD sites are simultaneously occupied by immobilized dye, i.e. bivalency occurs. Since the individual NAD sites on alcohol dehydrogenase are on opposite sides of the enzyme, bivalent binding would require an irregular accessible matrix surface such as that of cross-linked Sepharose, as opposed to a smooth accessible matrix surface. We have analyzed these zonal chromatographic results using eqn. 2 in two different manners. If we assume that all the enzyme is retained bivalently, then the dissociation constants for the enzyme—immobilized dye complexes are 10.1 and 22.1 μM. However, if we allow the occurrence of a non-integer valence and assume that the individual dissociation constants are identical, the data can be well fit to a valence of 1.6 and dissociation constants of 5.4 μM.

DISCUSSION

The results described above are summarized in Table I. It should be noted that the dissociation constant for the monovalent complex using the 1.38 m*M* immobilized-dye column, 5.3 μM, is within the error range of the value obtained by catalytic inhibition measurements. Such consistency, which

TABLE I

COMPARATIVE VALUES FOR ALCOHOL DEHYDROGENASE–DYE INTERACTION

Dye	Valence	Dissociation constant(s) (μM)
Free	1.0	4.5
Immobilized (1.38 mM)	1.0	5.3
Immobilized (11.6 mM)	1.6	5.4, 5.4
	2.0	10.1, 22.1

was also noted using lactate dehydrogenase, suggests that immobilization of the dye does not perturb its complexation with proteins in general and that the structure of the free dye–alcohol dehydrogenase complex established by crystallographic measurements probably pertains to complexes with the immobilized dye as well. These observations reinforce the perception that the anthraquinone ring of the dye provides the principal binding interaction with the protein and that remainder of the immobilized dye molecule including the triazine ring functions as a spacer to extend the terminal anthraquinone ring from the Sepharose surface. As also observed using lactate dehydrogenase [1] and a variety of other proteins [12], less than 5% of the immobilized dye is accessible to protein for complexation. Very recent studies [12] indicate that the accessibility of immobilized dye is controlled by both the ionic strength and the lyotropic nature of the components in the equilibration solvent, particularly the buffer salt.

Analysis of the zonal chromatographic results obtained with the maximally substituted Blue-Sepharose column suggests that the majority of enzyme is retained by complexation of immobilized dye with each of the two NAD sites on a given dimeric protein molecule. Computer fitting indicates that if all the enzyme–immobilized dye interaction is bivalent, then the affinity of the enzyme for immobilized dye is weaker than that observed for monovalent binding or for that of the free dye. By contrast, if the valence is allowed to have a non-integral value, the fitted dissociation constants are very similar to those observed for monovalent binding and for binding the free dye as shown in Table I. We suggest that this latter consideration may better represent a description of the chromatography in that the steric distribution of immobilized dye may preclude bivalent binding of all enzyme molecules at all times during the progress of the enzyme zone down the column. If this be correct, then the virtual identity of the dissociation constants for the enzyme–immobilized dye complexes in monovalent and divalent situations suggests that little cooperativity accompanies bivalency. By contrast, Hogg and Winzor [14] using recycling equilibrium measurements and a entirely different form of analysis find that the interaction between alcohol dehydrogenase and Blue-Sepharose can be treated as entirely bivalent and that the value for K_2 is about three-fold greater than that of K_1, indicating a modest degree of positive cooperativity. While we are not persuaded that cooperative binding is necessarily in evidence during zonal chromatography, it should be noted that both investigations support the view that alcohol dehydrogenase can bind bivalently to Blue-Sepharose.

ACKNOWLEDGEMENTS

This investigation was supported by a Public Health Services Research Grant No. GM22109 from the Institute of General Medical Sciences. We wish to thank William Shalongo for his assistance in facilitating the computer-assisted analysis of the data presented.

REFERENCES

1 Y.C. Liu, R. Ledger and E. Stellwagen, J. Biol. Chem., 259 (1984) 3796.
2 J.-F. Biellmann, J.-P. Samama, C.-I. Branden and H. Eklund, Eur. J. Biochem., 102 (1979) 107.
3 D.J. Cannon and R.H. McKay, Biochem. Biophys. Res. Commun., 35 (1969) 403.
4 B.L. Vallee and F.L. Hoch, Proc. Natl. Acad. Sci. U.S.A., 41 (1955) 327.
5 S.T. Thompson and E. Stellwagen, Proc. Natl. Acad. Sci. U.S.A., 73 (1976) 361.
6 L.A. Heyns and P. De Moor, Biochem. Biophys. Acta, 358 (1974) 1.
7 L.A. Haff and R.L. Easterday, in P.V. Sundaran and J. Eckstein (Editors), Theory and Practice in Affinity Techniques, Academic Press, New York, 1978, p. 23.
8 G.K. Chambers, Anal. Biochem., 83 (1977) 551.
9 H.W. Hethcote and C. DeLisi, J. Chromatogr., 248 (1982) 183.
10 B. Dunn and I.M. Chaiken, Biochemistry, 14 (1975) 2343.
11 D. Eilat, I.M. Chaiken and W.M. McCormack, Biochemistry, 18 (1979) 790.
12 Y.C. Liu, Thesis, University of Iowa, Iowa City, IA, 1985.
13 K. Dalziel, J. Biol. Chem., 238 (1963) 2850.
14 P.J. Hogg and D.J. Winzor, Arch. Biochem. Biophys., 240 (1985) 70.