ANTRACHINON-TRIAZINE DERIVATIVES OF POLYSACCHARIDES. AFFINITY OF BOVINE HEART LACTATE DEHYDROGENASE TO CIBACRON BLUE DERIVATIVES OF WATER-SOLUBLE DEXTRAN FRACTIONS AND HYDROXYETHYL-STARCHES

Jana BARTHOVÁ[a], Marie TICHÁ[a], Peter GEMEINER[b] and Danica MISLOVIČOVÁ[b]

[a] *Department of Biochemistry, Charles University, 128 40 Prague 2 and*
[b] *Institute of Chemistry, Slovak Academy of Sciences, 842 38 Bratislava*

Received February 25th, 1983

The interaction of lactate dehydrogenase with high-molecular-weight derivatives of Cibacron Blue was followed by measuring the reaction kinetics and by means of affinity electrophoresis. The dye was covalently bound to dextrans with relative molecular mass ranging from 5 000 to 2 000 000 and to hydroxyethyl-starches with relative molecular mass from 130 000 to 2 000 000. In the case of dextran derivatives of Cibacron Blue, various degrees of substitution by the dye were tested. Measurement of kinetics showed that the Cibacron Blue derivatives competed with NADH for the binding site on the enzyme and that when the dye was bound to a polysaccharide, the affinity of lactate dehydrogenase to the dye decreased. Both methods used confirmed that the strength of interaction did not depend on the relative molecular mass of the polysaccharide carrier or on the degree of substitution. The interaction of lactate dehydrogenase with hydroxyethyl-starch derivatives of Cibacron Blue was weaker than with dextran derivatives.

Considerable attention has been paid to the interaction of triazine dyes with proteins containing the so-called dinucleotide fold, and to making use of the phenomenon for the isolation of the proteins by affinity chromatography[1-3]. The character of the interaction of the dye with several proteins has already been investigated[4,5]. In our previous work[6,7], we studied the importance of the nature and molecular mass of the high-molecular-weight matrix for the interaction with lactate dehydrogenase (L-lactate: NAD^+ oxidoreductase EC 1.1.1.27). The present work is a contribution to this problem.

EXPERIMENTAL

Material

The lactate dehydrogenase preparation used was obtained by 60% saturation of the water extract of bovine heart muscle with ammonium sulphate.

Cibacron Blue 3G-A (C.I.Reactive Blue 2) derivatives of series T dextrans (Pharmacia, Uppsala) and waxy maize hydroxyethyl-starches DS 0·64—0·67 (a kind gift from Dr K. Granath of Pharmacia, Uppsala) were prepared by the reaction of the dye with the polysaccharides in aqueous alkaline medium, in the case of dextrans at 80°C (ref.[6]), in the case of hydroxyethyl-starches at 70°C (ref.[7]). The polysaccharide derivatives of the dye were purified and the homogeneity of the preparations was checked as described in the previous communication[6].

Methods

Measurement of lactate dehydrogenase activity. As a measure of enzyme activity, we determined the rate of NADH oxidation as followed by spectrophotometry at a wave length of 340 nm using a Pye-Unicam SP 8—400 spectrophotometer with a programmable calculator HP 97S. The reaction mixture contained 0·3 mol/l phosphate buffer, pH 7·5, $6{\cdot}3 \cdot 10^{-4}$ mol/l sodium pyruvate, and NADH at four different concentrations in the range of $4 \cdot 10^{-5}$ to $2 \cdot 10^{-4}$ mol/l. In studies of inhibition, Cibacron Blue or its derivatives was added. The reaction was started by adding the enzyme to the reaction mixture. Changes of absorbance were measured at 20 s intervals; five readings were taken, the first 20 s after starting the reaction. The computer was used for determining the initial reaction rate from the measured values. The values of Michaelis and inhibition constants were determined graphically and by calculation using the Lineweaver–Burk plot of the Michaelis-Menten equation.

The concentration of Cibacron Blue in free form or bound in high-molecular-weight derivatives was determined spectrophotometrically at a wave length of 610 nm, assuming the molar absorption coefficient to be equal to $13{\cdot}6 \cdot 10^3$ mol^{-1} . 1 . cm^{-1} (ref.[4]).

Affinity electrophoreis of lactate dehydrogenase was arranged as discontinuous electrophoresis in alkaline medium according to the standard procedure described by Davis[8], using only small pore gel. Affinity gels were prepared by adding a solution of Cibacron Blue Dextran to the polymerization mixture in such an amount that resulted in the final concentration of the dye in the range of $1{\cdot}0$ to $8{\cdot}0 \cdot 10^{-5}$ mol/l. The dye concentration was determined in the same way as when reaction kinetics were measured. Enzyme samples (80 μg) in 20% glycerol (20 μl) were applied to each tube and electrophoresis proceeded for 1·5 to 2 h at a current intensity of 4 mA per tube. The gels were stained specifically[9]. The distance of the zones from the start (d) was measured with ±0·5 mm accuracy. For each type of macromolecular derivative, 4 concentrations of immobilized dye were used ($c_i = 1{\cdot}0—8{\cdot}5 \cdot 10^{-5}$ mol/l); the measurements were performed on two gels for each concentration of immobilized dye. The dissociation constants of the lactate dehydrogenase— immobilized dye complex (K) were determined graphically from the dependence of $1/(d_0 - d)$ on $1/c_i$, where d_0 was distance of the protein zone from the start in the control gel without dye, d was the distance of the protein zone from the start in the affinity gel at a given concentration c_i of the immobilized dye; the point of intersection of the correlation line with the axis $1/c_i$ corresponds to the $-1/K$ value (ref.[10,11]).

RESULTS

Lactate dehydrogenase from bovine heart muscle was inhibited by free Cibacron Blue as well as by its macromolecular derivatives, namely dextran and hydroxyethyl--starches with covalently bound dye in their molecule. The inhibition was always competitive with respect to the coenzyme NADH. Table I presents the values of

inhibition constants that are mean values from two measurements of kinetics with two different inhibitor concentrations. It also states the values of dissociation constants of three of complexes lactate dehydrogenase isoenzyme with the immobilized dye; the results were obtained by affinity electrophoresis using dextran derivatives of Cibacron Blue. Electrophoresis could not be used for determining the dissociation constants of dextrans with a relaitve molecular mass of 5 and 10 thousand due to the fact that these compounds are mobile during electrophoresis owing to their small size. The results show that the free dye is the strongest inhibitor. The binding of Cibacron Blue to dextran somewhat decreased its affinity to the enzyme. The strenght of the interaction between the enzyme and the macromolecular inhibitor is not greatly influenced by the size of the dextran molecule. This fact is apparent from the results of kinetic measurement as well as from the values of dissociation constants determined by means of electrophoresis (Table I). The degree to which dextran with a relative molecular mass of 70 000 was substituted by the dye did not significantly influence the values of inhibition and dissociation constants (Table II).

The binding of Cibacron Blue to dextran decreased the affinity of the dye to the enzyme (Table I). It was of interest therefore to investigate the interaction of lactate dehydrogenase with Cibacron Blue bound to another type of polysacharide. Experiments were performed with hydroxyethyl-starch derivatives of Cibacron Blue with a relative molecular mass of 127 000, 402 000 and 1 902 000. Kinetic measurements

I TABLE I

nteraction of Cibacron Blue and Cibacron Blue dextrans with lactate dehydrogenase from bovine heart muscle. CB Cibacron Blue, CBD Cibacron Blue dextran (the numerals state the relative molecular mass of the polysaccharides). K dissociation constant of the enzyme–inhibitor complex determined by means of affinity electrophoresis, K_i inhibition constant determined by kinetic measurements

Inhibitor	Degree of substitution μmol/CB/g	$K_i \cdot 10^{-5}$ mol/l	$K \cdot 10^{-5}$ mol/l isoenzyme		
			I	II	III
CB	—	0·9	—	—	—
CBD-5 000	50·1	2·1	—	—	—
CBD-10 000	63·1	2·3	—	—	—
CBD-40 000	67·9	7·7	5·7	4·6	1·9
CBD-70 000	77·0	4·2	3·7	2·6	2·0
CBD-500 000	67·9	3·9	3·1	2·8	2·1
CBD-2 000 000	67·2	3·0	3·3	2·8	1·9

showed that the inhibition constants were 3 to 5 times higher than the inhibition constants of Cibacron Blue dextrans. The values of the dissociation constants of the complex of lactate dehydrogenase with hydroxyethyl-starch Cibacron Blue, determined by affinity electrophoresis, were 2 to 3 times higher than the values corresponding to the dextran derivatives. Unsubstituted polysacharides did not affect enzyme activity, nor did they influence electrophoretic mobility at the concentrations used.

DISCUSSION

In our previous work[6], we used rabbit muscle lactate dehydrogenase purchased from Boehringer. We did not succeed in determining the character of the inhibition or inhibition constants in kinetic measurements performed with the enzyme preparation. As we discovered later on by means of electrophoresis, the preparation contained at least 4 isoenzymes that differed significantly in the way they were composed of H and M subunits. The interaction of the subunits with triazine dyes differs[4,12] and this may be the reason why our kinetic measurements yielded results that were difficult to evaluate.

In order to investigate the interaction of lactate dehydrogenase with macromolecular derivatives of triazine dyes, we prepared the enzyme ourselves from bovine heart muscle. Our preparation also contained three isoenzymes, but electrophoresis showed that 80% was formed by the H subunit. Apart from kinetic measurements, we also determined the dissociation constants of the enzyme–inhibitor complex using affinity electrophoresis[11,13] which allowed the determination of these constants for the individual isoenzymes separately.

TABLE II

Influence of the substitution degree of dextran by the dye on the interaction of the inhibitor with bovine heart lactate dehydrogenase. The relative molecular mass of the dextran used was 70 000. K dissociation constant of the enzyme–inhibitor complex determined by affinity electrophoresis, K_i inhibition constant determined by kinetic measurements, CB Cibacron Blue

Degree of substitution μmol/CB/g	$K_i \cdot 10^{-5}$ mol/l	$K \cdot 10^{-5}$ mol/l isoenzyme		
		I	II	III
14·4	3·3	3·7	3·5	1·7
59·0	3·8	3·1	2·7	2·1
143·0	1·8	3·9	3·3	2·1

The results obtained by the two methods showed that inhibition of lactate dehydrogenase by the dye was competitive to NADH. Triazine dyes may therefore be assumed to have a structural arrangement similar to the coenzyme. The inhibition and dissociation constants of the enzyme–inhibitor complex, calculated from results of our experiments with Cibacron Blue dextrans, agree with the data published[4,13] on that complex of the commercial preparation of Blue Dextran from Pharmacia with heart actate dehydrogenase.

The kinetic determination of the inhibition constants allowed us to compare the affinity of free Cibacron Blue with the affinity of macromolecular derivatives of the dye. The values obtained in experiments with Cibacron Blue bound to dextrans and to the branched α-glucane (hydroxyethyl-starch) give eivdence that the addition of the macromolecular component to the dye decreased its affinity to the enzyme. Although we tried a wide scale of dextrans with relative molecular weight ranging from 5 thousand to 2 million, neither of the methods used revealed any influence of the size of the molecule on the affinity of inhibitor to the enzyme. The hydroxyethyl--starch derivative of Cibacron Blue had a lower affinity to lactate dehydrogenase than dextrane derivatives. The two types of polysaccharides used (dextran and amylopectin) differ considerably in the main and side glycoside bonds, in the branching of the side chains and in the regularity of the branching[14–16]. These differences are the reason for different conformations of the α-glucans in solution.

The results suggest that the binding site for triazine dyes is located on the enzyme in such a way that even a dextran molecule with a relative molecular weight of 5 000 can create certain steric hindrance. It is known that the triazine dyes are bound to the enzyme at the coenzyme binding site. The site is located in a relatively deep pocket in lactate dehydrogenase, according to Holbrook and coworkers[17]. Our results corroborate this conception of the structure of the coezyme binding site of the active center of lactate dehydrogenase.

REFERENCES

1. Clonis Y. D., Lowe C. R.: Biochim. Biophys. Acta *659*, 86 (1981).
2. Leden D. J., Feldhoff R. C., Chan S. K.: Biochem. J. *205*, 331 (1982).
3. Hughes P., Sherwood R. F., Lowe C. R.: Biochem. J. *205*, 453 (1982).
4. Thompson S. T., Stellwagen E.: Proc. Nat. Acad. Sci. U.S.A. *73*, 361 (1976).
5. Edwards R. A., Woody R. W.: Biochemistry *18*, 5197 (1979).
6. Gemeiner P., Mislovičová D., Zemek J., Kuniak L.: This Journal *46*, 419 (1981).
7. Gemeiner P., Mislovičová D., Šoltés L., Barthová J., Tichá M.: Unpublished results.
8. Davis B. J.: Ann. N. Y. Acad. Sci. *121*, 404 (1964).
9. King J.: Can. J. Bot. *48*, 533 (1970).
10. Hořejší V.: J. Chromatogr. *178*, 1 (1979).
11. Tichá M., Barthová J., Labský J., Semanský M.: J. Chromatogr. *194*, 183 (1980).
12. Wilson J. E.: Biochem. Biophys. Res. Commun. *72*, 816 (1976).

13. Tichá M., Hořejší V., Barthová J.: Biochim. Biophys. Acta *534*, 58 (1978).
14. Lindberg S., Svensson S.: Acta Chem. Scand. *22*, 1907 (1968).
15. Aspinall G. O., Stephen A. M. in the book: *Organic Chemistry, Series One* (D. H. Hey, Ed.), Vol. 7 — Carbohydrates (G. O. Aspinall, Ed.), p. 285. Butterworths, London 1973.
16. *Dextran Fractions, Dextran Sulphate, DEAE Dextran* — defined polymers for biological research, Leaflet of Pharmacia Fine Chemicals, Uppsala, 1977, p. 5.
17. Holbrook J. J., Liljas A., Steindel S. J., Rossman M. G. in the book: *The Enzymes* (P. D. Boyer, Ed.), p. 240, Vol. 11, Acad. Press, New York 1975.

Translated by L. Servítová.