Journal of Chromatography, 194 (1980) 183–189
Elsevier Scientific Publishing Company, Amsterdam — Printed in The Netherlands

CHROM. 12,740

DETERMINATION OF THE INTERACTION OF LACTATE DEHYDROGENASE WITH HIGH-MOLECULAR-WEIGHT DERIVATIVES OF AMP BY AFFINITY ELECTROPHORESIS

M. TICHÁ and J. BARTHOVÁ*
Department of Biochemistry, Charles University, Albertov 2030, 128 40 Prague 2 (Czechoslovakia)
J. LABSKÝ
Institute of Macromolecular Chemistry, Czechoslovak Academy of Sciences, 162 06 Prague 6 (Czechoslovakia)
and
M. SEMANSKÝ
Department of Biochemistry, Charles University, Albertov 2030, 128 40 Prague 2 (Czechoslovakia)
(Received February 5th, 1980)

SUMMARY

The interaction of lactate dehydrogenase with high-molecular-weight derivatives of AMP was studied by affinity electrophoresis in an alkaline buffer system and by means of kinetic measurements. AMP was coupled to synthetic hydroxypropylmethacrylamide copolymers through glycine, 6-aminohexanoic and 12-aminododecanoic spacer arms. The values of the dissociation constants (K) of the lactate dehydrogenase isoenzymes–immobilized AMP complexes determined by affinity electrophoresis decreased with increasing length of the spacer arm. Lactate dehydrogenase was competitively inhibited by high-molecular-weight derivatives of AMP; values of the inhibition constants (K_i) also depended on the spacer arm: the longer the spacer arm the stronger was the interaction between the enzyme and the inhibitor. K_i values for high-molecular-weight derivatives of AMP were lower than those obtained for free AMP.

INTRODUCTION

Affinity electrophoresis is a type of electrophoretic separation of proteins based on their different affinities for ligands immobilized in the separating gel. This method represents a convenient tool for the quantitative study of the interaction of proteins with immobilized and free ligands.

So far, affinity electrophoresis in polyacrylamide gels has been used for the study of the interaction of phosphorylases with glycogen[1,2], lectins[3–6], α-galactosidase[7] and antibodies[8] with immobilized and free sugars and trypsin with immobilized *p*-aminobenzamidine derivatives[9]. Affinity electrophoresis in polyacrylamide gel has been also used for the qualitative and quantitative study of the interaction of several

0021-9673/80/0000–0000/$02.25

enzymes, mostly possessing a dinucleotide fold in their structure, with Blue Dextran[10]. Dissociation constants of complexes of lactate dehydrogenase with immobilized Cibacron Blue obtained using affinity electrophoresis were in good agreement with the inhibition constants of the enzyme and Blue Dextran obtained by kinetic measurements[11,12], as well as with association constants of enzyme–Cibacron Blue complexes determined using differential spectroscopy[13]. However, Blue Dextran as a group-specific affinity medium interacts both with enzymes containing a dinucleotide fold and with some other proteins[10]. The possibility of applying affinity media containing more specific ligands for the study of protein–ligand interactions is in some instances more advantageous, using both affinity chromatography and affinity electrophoresis.

For the affinity chromatography of lactate dehydrogenase, immobilized NAD or part of its molecule are currently used[14]. In this work the applicability of affinity electrophoresis for the quantitative study of interactions of the enzyme with its immobilized inhibitor was employed. The data obtained characterizing the binding parameters were compared with the inhibition constants of lactate dehydrogenase by high-molecular-weight derivatives of AMP and with those of free AMP.

EXPERIMENTAL

Bovine heart lactate dehydrogenase was prepared by the precipitation of an aqueous extract of heart muscle with ammonium sulphate to 65% saturation and, after dialysis, was lyophilized. AMP was purchased from Reanal (Budapest, Hungary).

Preparation of AMP coupled to hydroxypropylmethacrylamide copolymer

Copolymers of the 4-nitrophenyl ester of N-methacroyl derivatives of ω-amino acids were prepared as described by Labský and Kálal[15,16]. The molecular weight of the polymers was determined by the light-scattering method.

Coupling of AMP to these polymers was performed by heating the polymer (300 mg) with AMP (100 mg) in 3 ml of dimethyl sulphoxide in a closed vessel at 50 °C for 5 h. The resulting polymer derivative was dialysed exhaustively against 50% ethanol. The dialysed solution was evaporated to dryness, the residue was dissolved in methanol (2 ml) and the AMP derivative was precipitated with acetone (30 ml).

Affinity electrophoresis

Polyacrylamide gel electrophoresis was performed using the apparatus designed by Davis[17] in a discontinuous alkaline buffer system[18] according to the standard procedure (omitting large-pore gel layers).

Protein samples (80 μg) in 20% glycerol solution (20 μl) were applied to each tube (5 × 75 mm) and electrophoresis was run at 4 mA per tube for 1.5–2 h. Gels were stained specifically[19]. The migration distances of the zones of lactate dehydrogenase isoenzymes were measured with an acuracy $\pm$ 0.5 mm.

The dissociation constants (K) of the complexes of the lactate dehydrogenase isoenzymes and immobilized AMP were obtained by a modification of our original method[3,9]. The values of $1/d_0 - d$ were plotted against $1/c_t$ (d_0 = mobility on control gel, d = mobility on an affinity gel containing a molar concentration c_t of immobilized ligand). The straight line yields $-1/K$ as the intercept with the abscissa.

Affinity gels were prepared by addition of an appropriate amount of the solu-

tion of AMP-containing polymer to the polymerization mixture to give a desired concentration c_i of immobilized enzyme inhibitor; c_i was used in the range $3.2 \cdot 10^{-5}$–$6.4 \cdot 10^{-4}$ *M*.

The AMP concentration in the polymer solution was determined spectrophotometrically at 259 nm using a molar extinction coefficient of 15.4 $mmol \cdot l^{-1} \cdot cm^{-1}$.

Measurement of the rate of the reaction catalysed by lactate dehydrogenase

The determination of the rate of reaction in the direction of lactate oxidation was based on the spectrophotometric measurement of the hydrazone formed in alkaline medium by pyruvate and 2,4-dinitrophenylhydrazine. The reaction mixture contained, in 0.6 ml of 0.1 *M* Tris–acetate buffer (pH 9.1), 0.12 mmole of sodium lactate, 0.5–2 μmole of NAD and an appropriate amount of inhibitor. The reaction was started by addition of 1 μg of enzyme dissolved in 50 μl of buffer. The type of inhibition and values of the inhibition constants were determined both by the method of Lineweaver and Burk and that of Dixon.

RESULTS

Coupling of AMP to hydroxypropylmethacrylamide copolymer

By means of copolymerization of N-(2-hydroxypropyl)methacrylamide with 4-nitrophenyl esters of N-methacroyl derivatives of ω-amino acids, copolymers were obtained, in which the content of reactive 4-nitrophenyl esters was about 3 mole% (Fig. 1, $n = 1, 5, 11$; R = 4-nitrophenyl residue). The molecular weight of the prepared copolymers was 37,000 for $n = 5$ and 11 and 42,000 for $n = 1$.

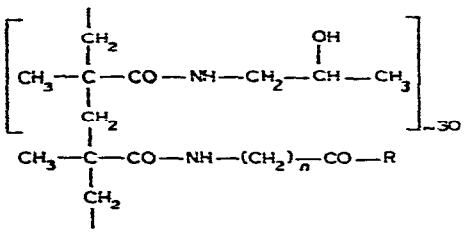

Fig. 1. Structure of AMP derivatives. R: (A) AMP immobilized through the 6-amino group of adenine; (B) $-NH(CH_2)_5CH_3$.

For coupling of AMP to soluble polymers, the reaction of 4-nitrophenyl esters with the 6-amino group of AMP was employed. AMP was bound to the polymer through different spacer arms (Fig. 1, R = A, $n = 1, 5, 11$). The same reaction was used for the preparation of polymer containing coupled hexylamine (Fig. 1, R = B, $n = 5$).

Affinity electrophoresis

The addition of hydroxypropylmethacrylamide copolymers containing coupled AMP to polyacrylamide gels caused a decrease in the electrophoretic mobility of lactate dehydrogenase isoenzymes in comparison with control gels. The decrease in electrophoretic mobility is dependent on the concentration of immobilized AMP in the polyacrylamide gels.

The control gels were prepared by an addition of hydroxypropylmethacrylate copolymer containing no AMP to polyacrylamide gels. The presence of the copolymer without a ligand did not affect the mobility of lactate dehydrogenase isoenzymes, indicating no "non-specific" interaction of the polymer with the enzyme. To eliminate the possible effect of interaction of lactate dehydrogenase with the spacer arms, hydroxypropylmethacrylamide copolymer containing coupled hexylamine was tested. The presence of this alkyl polymer derivative in polyacrylamide gel in the same concentration as the polymer AMP derivatives did not affect the electrophoretic mobility of lactate dehydrogenase isoenzymes under the experimental conditions. Similar results were obtained with the alkyl derivatives of dextran which were prepared according to Nakamura *et al.*[20], used for the detection of hydrophobic interactions in the case of lactate dehydrogenase[21].

In the preparation of bovine heart lactate dehydrogenase, three isoenzymes were detected, which differ slightly in their interaction with immobilized AMP. The apparent dissociation constants of lactate dehydrogenase–immobilized AMP complexes calculated from the dependence of the electrophoretic mobility on AMP concentration are given in Table I.

TABLE I

DISSOCIATION CONSTANTS OF LACTATE DEHYDROGENASE ISOENZYMES AND IMMOBILIZED AMP COMPLEXES DETERMINED BY MEANS OF AFFINITY ELECTROPHORESIS

*Polymer**	*K(M)*		
	Isoenzyme 1	*Isoenzyme 2*	*Isoenzyme 3*
HPMA		No interaction observed	
HPMA-C_6-hexyl		No interaction observed	
HPMA-C_2-AMP		Weak interaction	
HPMA-C_6-AMP	$2.7 \cdot 10^{-3}$	$2.3 \cdot 10^{-3}$	$1.9 \cdot 10^{-3}$
HPMA-C_{12}-AMP	$3.9 \cdot 10^{-3}$	$3.7 \cdot 10^{-3}$	$2.7 \cdot 10^{-3}$

*HPMA = hydroxypropylmethacrylate polymer; C_2 = glycine residue; C_6 = 6-aminohexanoic acid residue; C_{12} = 12-aminododecanoic acid residue.

Kinetic measurement of inhibition of lactate dehydrogenase by AMP and its high-molecular-weight derivatives.

Free AMP and AMP coupled to hydroxypropylmethacrylamide copolymer through spacer arms of different length were found to decrease the rate of the reaction catalysed by lactate dehydrogenase from bovine heart muscle. The dependence of the reaction rate either on the concentration of coenzyme (NAD) or on the concentration of inhibitor indicated that all derivatives of AMP as well as free AMP acted as competitive inhibitors to coenzyme (Fig. 2). From these dependences the values of the inhibition constants were calculated (Table II). As can be seen, all high-molecular-weight derivatives of AMP were characterized by a higher affinity to the enzyme than free AMP; values of the inhibition constants were one order of magnitude lower for high-molecular-weight derivatives than for free AMP. Values of the inhibition constants depended on the length of the spacer arm. The lowest affinity of the enzyme was observed with the shortest spacer arm. The rate of the enzyme reaction was not influen-

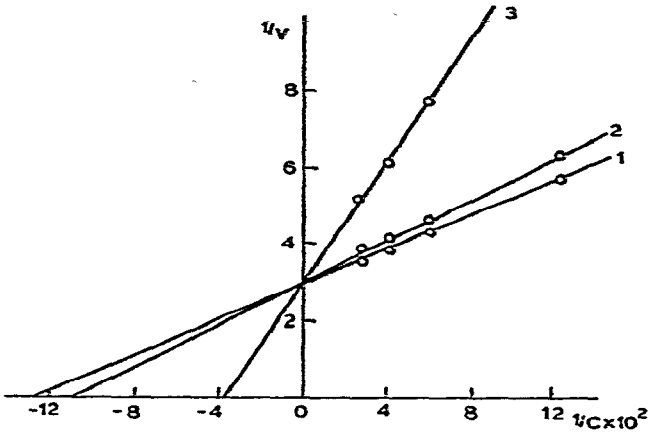

Fig. 2. Determination of inhibition constants for bovine heart lactate dehydrogenase and derivatives of AMP. 1, No inhibition; 2, inhibition by free AMP ($5.7 \cdot 10^{-4}$ *M*); 3, inhibition by HPMA-C_6-AMP (concentration of coupled AMP, $5 \cdot 10^{-4}$ *M*). *C* = concentration of NAD (*M*); *V* = rate of reaction (in International Units).

TABLE II

INHIBITION OF LACTATE DEHYDROGENASE FROM BOVINE HEART MUSCLE BY AMP AND ITS HIGH-MOLECULAR-WEIGHT DERIVATIVES

Values of K_i determined from kinetic measurements.

*Inhibitor**	K_i (*M*)
AMP	$5.4 \cdot 10^{-3}$
HPMA-C_2-AMP	$5.9 \cdot 10^{-3}$
HPMA-C_6-AMP	$2.0 \cdot 10^{-4}$
HPMA-C_{12}-AMP	$1.0 \cdot 10^{-4}$
HPMA	No inhibition
HPMA-C_6-hexyl	No inhibition

*HPMA = hydroxypropylmethacrylate polymer; C_2 = glycine residue; C_6 = 6-aminohexanoic acid residue; C_{12} = 12-aminododecanoic acid residue.

ced by hydroxypropylmethacrylamide copolymer alone, or by this copolymer containing coupled hexyl residues.

DISCUSSION

The results show that the strength of the interaction of lactate dehydrogenase with derivatives of AMP increased with increasing spacer arm length from C_2 to C_6, whereas almost no difference was observed between C_6 and C_{12} spacer arms. These observation are in a good agreement with the results of Hipwell *et al.*[22] on the affinity chromatography of lactate dehydrogenase on homologous series of immobilized N^6-aminoalkyl-AMP. This phenomenon could be explained by an improved steric accessibility of the ligand bound through a longer spacer arm and/or by the fact that the spacer arm contributes to the binding of the enzyme. However, our results have shown that high-molecular-weight derivatives containing coupled hexyl residues without AMP do not interact with the enzyme studied. The contribution of the spacer

arm to the binding of lactate dehydrogenase to AMP derivatives was also suggested by Hipwell *et al.*[22]

In a previous paper[10] we described the application of affinity electrophoresis to a quantitative study of the interaction of lactate dehydrogenase isoenzymes with immobilized Cibacron Blue. Dissociation constants of complexes of lactate dehydrogenase and immobilized Cibacron Blue determined by affinity electrophoresis agreed satisfactorily with values of inhibition constants (K_i) determined kinetically[11] and also with dissociation constants measured spectrophotometrically[13]. In the case of high-molecular-weight derivatives of AMP used in the present work, values of the dissociation constants of enzyme–immobilized inhibitor complexes determined by means of affinity electrophoresis are in good agreement with the constants of inhibition of lactate dehydrogenase by free AMP (our results and ref. 23) determined kinetically. However, interesting data were obtained from kinetic measurements of the inhibition of lactate dehydrogenase by AMP derivatives coupled to hydroxypropylmethacrylamide copolymer. In this instance inhibition constants were about by one order of magnitude lower than with free AMP. This fact could be explained by non-biospecific interference of the spacer arms and/or of hydroxypropylmethacrylamide copolymer, which could contribute to the binding of the inhibitor to the enzyme, even though hydroxypropylmethacrylamide copolymer alone or with a coupled spacer arm does not interact with the protein and does not affect its enzyme activity. Such non-biospecific contributions of the spacer arm are known to be affected by the nature of a spacer arm and by the composition of buffer solution used, mainly by ionic strength[24].

The difference between the dissociation constants of enzyme–immobilized inhibitor complexes determined by affinity electrophoresis and constants of inhibition of the lactate dehydrogenase activity by high-molecular-weight derivatives of the inhibitor might be attributed to several factors: (i) the conditions for the determination of inhibition constants by kinetic measurements differed from those in the determination of dissociation constants by affinity electrophoresis. (ii) Kinetic measurements of inhibition constants were performed in the presence of the substrate lactate, whereas in affinity electrophoresis only the interaction of lactate dehydrogenase with immobilized AMP was followed; the presence of the second substrate might influence the binding of inhibitor. Analogous phenomena were described for the precipitation of lactate dehydrogenase with a bifunctional NAD coupound; affinity precipitation of the enzyme occurred only in the presence of substrate[25]. This fact is also used for the specific elution of lactate dehydrogenase from an affinity column[14]. (iii) Interaction of lactate dehydrogenase with immobilized AMP under the conditions of affinity electrophoresis could be stronger than with free AMP, as was determined from kinetic measurements; however, higher values of the dissociation constants of enzyme–immobilized AMP complexes in comparison with inhibition constants determined kinetically might be also caused by the lower effective concentration of immobilized ligand in polyacrylamide gel than the analytical concentration used for the calculation of the dissociation constants.

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