THE BINDING-SITES OF RABBIT MUSCLE ALDOLASE*

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The binding of a potent competitive inhibitor of rabbit muscle aldolase, D-arabinitol-1,5-diphosphate-1-^{14}C, has been examined by equilibration against Bio-Gel P-6 in a column and by use of a partition-cell in the ultracentrifuge. All of the data obtained are consistent with aldolase having three binding sites for this compound.

Studies of the number of binding-sites for substrate in rabbit muscle aldolase are of interest because of the multiplicity of peptide chains in the protein (Stellwagen and Schachman (1962), Kawahara and Tanford (1966)) and the lack of agreement in the published data concerning the numbers of substrate molecules which are bound (Lai et al. (1965), Westhead et al. (1963)).

Two moles of dihydroxyacetone phosphate are bound per mole of enzyme by reduction of the ES complex with sodium borohydride (Lai et al. (1965)) whereas only one mole of dihydroxyacetone phosphate or of fructose-diphosphate appears to be bound when the binding is examined in the ultracentrifuge and by equilibrium dialysis (Westhead et al. (1963)).

MATERIALS AND METHODS D-arabinitol-1,5-diphosphate-1-^{14}C** was synthesized by the published method (Hartman and Barker (1965)) from D-arabinose-1-^{14}C purchased from the New England Nuclear Corporation. The compound was isolated as the cyclohexylammonium salt. It had a specific activity of 3.24 $\pm$ 0.1 x

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**D-arabinitol-1,5-diphosphate-1-^{14}C will be referred to hereafter as AP.

10^7 d. $min.^{-1}$ $mmole^{-1}$. Descending paper chromatography on Whatman 1 using ethanol:conc. ammonium hydroxide:water (6:2:2, v/v) showed the compound to be pure as judged by radioactivity measurements, phosphate spray and periodate-benzidine sprays.

Aldolase was purchased from Boehringer and had a specific activity of at least 10.0 when assayed by the method of Blostein and Rutter (1963).

Ultracentrifuge studies were performed in a Spinco Model E Ultracentrifuge using a moving partition cell at 24° and rotor speeds of 59,780 r.p.m. Centrifugation was carried out until the boundary was below the partition rest position (approximately 1 hour). Solutions were freshly prepared and mixed a few minutes prior to centrifugation when a 0.1 ml aliquot of the solution was removed for counting. After the sedimentation was completed a 0.1 ml aliquot of the solution above the boundary was taken for counting. The difference in c.p.m. between the two samples is a measure of the amount of inhibitor bound after correction for the sedimentation of the inhibitor. In Fig. 1 the data is plotted according to Scatchard (1959). Extrapolation to $\frac{N}{S} = 0$, i.e. infinite inhibitor concentration, indicates that three moles of inhibitor can be bound per mole of enzyme. The observed sedimentation coefficient was not affected by the presence of the inhibitor; S_{obs} = 7.54 (7.547 with inhibitor) in glycylglycine buffer, pH 7.5 at 21°.

Total Numbers of Binding Sites by Equilibration Against Bio-Gel P-6 (Column)

The technique is essentially that described by Hummel and Dreyer (1962). All of the solutions used were 1.5 x 10^{-2} M in glycylglycine at pH 7.5. COLUMN 1. A column of Bio-Gel* P-6 (0.8 x 100 cm) was equilibrated against 1.0 x 10^{-4} M AP at a flow rate of 0.4 ml/min. When the specific activity of the eluate equalled that of the eluent (i.e. 1470dpm/ml), 5.0 ml of a 1.68 x 10^{-5} M solution of aldolase, 1.0 x 10^{-4} M in AP, was added to the column and eluted with the same solution used to equilibrate the column. Care was taken to minimize mixing between the sample and the eluent during addition of the sample to the column.

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The eluate was collected in 1.3 ml fractions and assayed for AP and protein. Radioactivity was measured using a Nuclear Model 720, Liquid Scintillation Counter by adding 1.0 ml of eluate to 15 ml of Bray's solution and 1.0 ml of hydroxide of hyamine 10X and counting for a length of time sufficient to decrease the probable error to less that 1%. Corrections were made for the effect of protein on the counting efficiency. Protein concentrations were measured at 280 mμ, assuming a value of 0.938 for $E^{280}_{1\%}$ for aldolase (Donovan (1964)). The results are given in Table 1.

Experiments performed with the various concentrations of enzyme and of AP used to equilibrate the column all indicated that 3.0 $\pm$ 0.4 moles of AP are bound per mole of enzyme.

COLUMN 2. To a column of Bio-Gel P-6 (0.8 x 100 cm) equilibrated with 5.6×10^{-5} M AP was added 5 ml of a solution 1.2×10^{-5} M in aldolase and 9.2×10^{-5} M in AP, i.e., sufficient AP to saturate three binding sites and to make the solution 5.6×10^{-5} M with respect to free AP. Elution with 5.6×10^{-5} M AP gave a peak of radioactivity coincident with a peak of protein concentration. There was no indication of a trough following the peak (see Table 1).

DISCUSSION When the binding of inhibitor is estimated from the ultracentrifuge data plotted according to Scatchard (1959) the number of binding sites is clearly three. Since there is no curvature apparent in the plot the binding sites appear to be independent of one another and to have dissociation constants of 5×10^{-6} M. The effect of the value given to the molecular weight of aldolase on the number of moles of inhibitor bound is slight when this method is used.

Equilibration against Bio-Gel, as used in this work, should give high values for binding since the column acts as an "infinite" source of inhibitor against which the enzyme is equilibrated with the result that even relatively weak binding will be detected. To differentiate between strong and weak binding sites would require that much more concentrated solutions of enzyme be equilibrated against much more dilute solutions of inhibitor. Unfortunately the

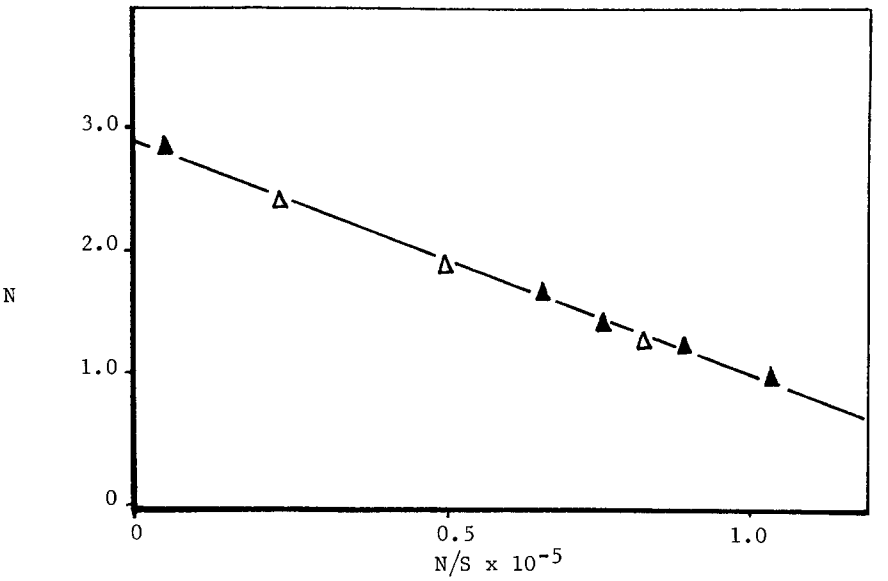

Fig. 1. The binding of D-arabinitol-1-^{14}C-diphosphate to aldolase at 24° in .015 M glycylglycine buffer pH 7.5; N is the number of moles of inhibitor bound per mole of enzyme; S is the concentration of free inhibitor. ▲ = 2.2 x 10^{-5} M aldolase, Δ = 2.9 x 10^{-5} M aldolase calculated for molecular weight of 142,000 (Stellwagen and Schachman, 1962). Data calculated using a molecular weight of 158,000 (Kawahara and Tanford, 1966) give a line with the same slope which extrapolates to 3.1 sites.

specific activity of the latter is not sufficiently high to allow this to be done with precision. The error possible in the data presented is high because of the influence of protein on counting efficiency and the fact that relatively small counting errors have a large effect on the calculated ratio of inhibitor to enzyme. The results clearly indicate that there are at least three binding sites per molecule with the possibility that at very high inhibitor levels other, perhaps non-specific, binding can occur. The effect of the value given to the molecular weight of aldolase on the number of binding sites found by this method is much more pronounced.

The previous findings of one site for fructose diphosphate may indicate that the enzyme in the presence of its substrate is undergoing conformational changes which are inherent in its catalytic function and which preclude its binding more than one molecule at a time whereas with the inhibitor a confor-

TABLE 1

THE BINDING OF D-ARABINITOL-1-^{14}C DIPHOSPHATE TO ALDOLASE AS MEASURED BY EQUILIBRATION AGAINST BIO-GEL P-6

	COLUMN #1			COLUMN #2	
Tube	excess μmoles AP per ml (±2.0) x10^{-2}	μmoles of aldolase per ml (±0.2)* x10^{-2}	AP/E* (±0.2)	excess μmoles AP per ml (±2.0) x10^{-2}	AP/E* (±0.2)
11	0	0		0	
12	+2.42	0.78	3.09	+2.62	2.79
13	+5.73	1.70	3.36	+3.58	2.98
14	+5.79	1.76	3.28	+3.80	3.17
15	+6.67	1.92	3.47	+3.18	2.87
16	+4.12	1.40	2.94	+1.46	3.0
17	-1.21	0		0	
18	-2.43	0		0	
19	-3.18			0	
20	-4.79			0	
21	-4.85			0	
22	-3.91			0	
23	-2.70			0	
24	-0.61			0	
25	0			0	

*Calculated on basis of MW 142,000 for aldolase. When MW 158,000 for aldolase is used slightly higher ratios of AP/E are obtained.

mationally stable complex is produced. Although Westhead et al. (1963) used ratios of substrate to enzyme of 1.15 to 1.0 and smaller there was sufficient substrate present for more than one molecule to have been bound per molecule of enzyme if the binding constants for subsequent molecules were of the order of 1 x 10^{-5} or smaller.

The reductive coupling of 2 molecules of dihydroxyacetone phosphate per molecule of enzyme has been interpreted by Lai et al. (1965) as indicating two active sites in aldolase. However the number of sites obtained by this method may be less than the maximum number since conformational changes may accompany the reduction.

Experiments to study the binding of this inhibitor in the presence of the various substrates of aldolase are in progress.

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