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BINDING OF NICOTINAMIDE-ADENINE DINUCLEOTIDE TO RABBIT MUSCLE ALDOLASE

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Summary

A direct interaction of rabbit muscle fructose-1,6-bisphosphate aldolase with NAD^+, NADH, and NAD-agarose was demonstrated. The electrostatic forces are primary involved in this interaction. Two specific binding sites for the dinucleotide were observed. One of them is located at the active site of the enzyme, the second is in a region of weak binding site for ATP and fructose 1,6-bisphosphate.

According to Stellwagen [1], rabbit muscle aldolase (D-fructose-1,6-bisphosphate D-glyceraldehyde-3-phosphate lyase, EC 4.1.2.13) has a locus of NAD-domain which can be predicted by sequence analysis using the method of Chou and Fasman [2]. Demonstration of Cibacron blue F3GA binding to aldolase lends further support to this proposal [3]. It has been postulated that the entire molecule of Cibacron blue F3GA mimics NAD^+, and its binding is specific for the topological folding in a supersecondary structure found in proteins, called a dinucleotide fold. The analogous conclusion concerning the dinucleotide binding site in aldolase might be inferred from the modification [4] and kinetic [5] studies.

On the other hand, Beissner and Rudolph [6]have shown that it is most likely that Cibacron blue F3GA is nonspecific and binds only when the catalytic site is large enough, irrespectively of substrate requirement or overall protein folding pattern. Bornmann and Hess [7]have suggested that hydrophobic interactions of the aromatic groups of Cibacron blue F3GA and its analogs contribute predominantly to the binding of these dyes to proteins. Since a possibility of a covalent modification of the proteins with Cibacron

Abbreviation: Mops, 3-(*N*-morpholino) propanesulfonate.

blue F3GA cannot be excluded [8], the results of kinetic studies should be interpreted with special care. Recently Grazi et al. [9] have shown that rabbit muscle aldolase binds tightly 3.5 molecules of this dye per subunit, too great a number to support the hypothesis that binding of Cibacron blue F3GA is a specific indication of the presence of NAD-domain. For the above reasons we have felt it might be of great importance to demonstrate a direct interaction of NAD^+ with aldolase and study this interaction. Affinity chromatography of native aldolase and its β-glycerylphosphate derivative on NAD-agarose column was the method of choice; the experimental details are given in the legends to Figs. 1 and 2.

Native aldolase can be bound tightly to NAD-agarose column in 10 mM Mops-Na buffer/0.2 mM EDTA, pH 7.5 (Fig. 1A), and only traces of aldolase activity were detected in the collected fractions when the column was developed with the buffer containing 20 mM NaCl (Fig. 1B). The bound enzyme could be eluted as a sharp peak by 100 mM NaCl (Fig. 1B). The influence of the increasing ionic strength on the aldolase elution profile indicates that electrostatic forces contribute significantly to the binding.

Because rabbit muscle aldolase is a rather basic protein with a p*I* of 8.5 [11], the question arose as to whether binding of this enzyme to NAD-agarose is specific to certain region of the enzyme molecule or occurs via any salt linkage between exposed amino or guanidine groups of the protein and an electronegative pyrophosphate moiety of NAD^+. The sharp displacement of the bound enzyme by 0.25 mM fructose-1,6-P_2 or ATP (Fig. 1C and 1D) provided the evidence that NAD^+ was bound specifically to the same region of the enzyme molecule as its substrate and competitive inhibitor [12]. Experiments showing that 8.5 mM NADH eluted aldolase from NAD-agarose column, whereas only partial elution was observed with 40 mM NAD^+ (Fig. 1E and 1F) indicate again that the net charge of interacting ligand is one of the most important factors involved. Assuming that the free energy of binding depends linearly on the net charge of dinucleotides in the same way as was found for mononucleotides [12], one may predict that dissociation constants for the complexes of aldolase with NADH and NAD^+ would be approximately 3 and 30 mM, respectively. These estimations are in accord with the above observations.

Seemingly unexpected results were obtained when phosphotrioses were employed for the elution of aldolase from the column. The affinity of these compounds to the enzyme is high [13] but neither dihydroxyacetone phosphate nor glyceraldehyde 3-phosphate was effective in the displacement of the native aldolase when used at a concentration of 0.25 mM (Fig. 1G and 1H). Partial elution of aldolase was observed with 0.85 mM concentration of dihydroxyacetone phosphate or glyceraldehyde 3-phosphate (Fig. 1G and 1H). Moreover, experiments with β-glycerylphosphate derivative of aldolase show that aldolase with dihydroxyacetone phosphate-subsite blocked is able to bind to NAD-agarose, although with the lower affinity in comparison with the native enzyme (Fig. 2A). For the elution of dihydroxyacetone phosphate-modified aldolase higher concentration of ATP (0.5 mM) or fructose-1,6-P_2 (1 mM) is required (Fig. 2C and 2D).

One of the possible explanations of the above results is that NAD^+ is able

to bind to the two subsites of aldolase active center (dihydroxyacetone phosphate and glyceraldehyde 3-phosphate). For steric reasons, when active center is filled up by fructose-1,6-P_2 or bulky ATP molecule, NAD^+ cannot be bound. Partial occupancy of the active center either by dihydroxyacetone phosphate or glyceraldehyde 3-phosphate allows NAD^+ to be bound to the

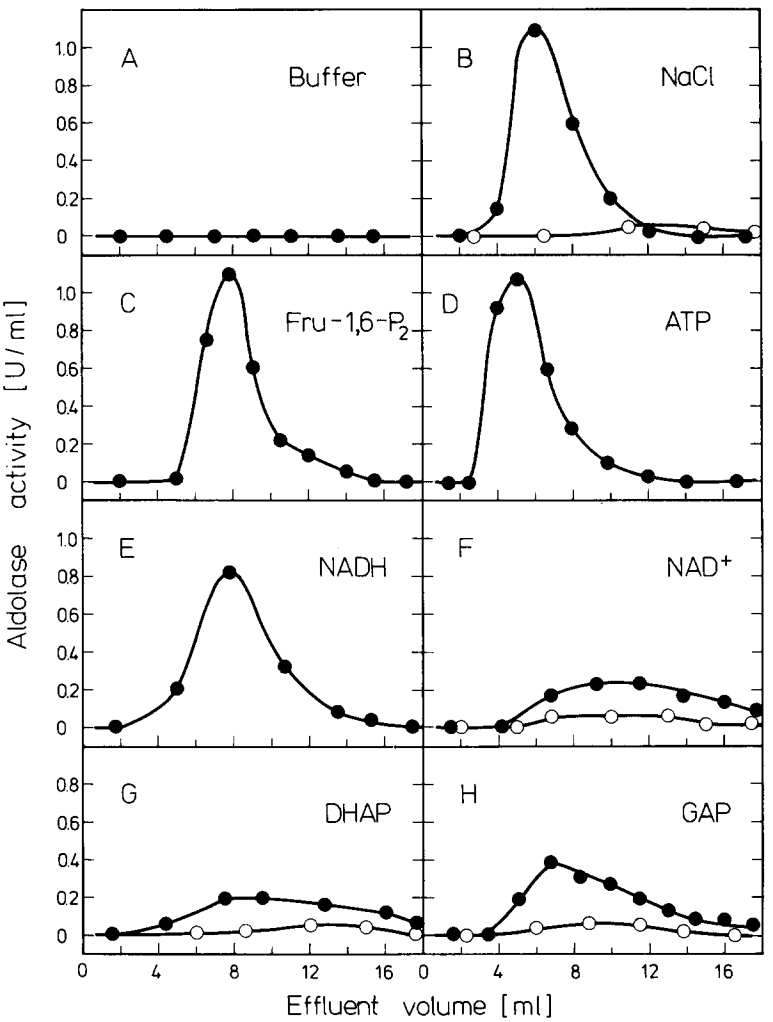

Fig. 1. Chromatography of native aldolase on NAD-agarose column. Rabbit muscle aldolase with the specific activity of 16 units/mg protein was prepared according to Penhoet et al. [10]. NAD-agarose was purchased from Sigma (catalogue No. N 6130). For all chromatography experiments 4.5 ml of NAD-agarose were placed in a 5 ml plastic syringe. The column was equilibrated with 10 mM Mops-Na buffer/0.2 mM EDTA, pH 7.5 and 0.25 ml of aldolase solution in the above buffer containing 0.34 mg of protein was applied on the top of the column. The column was washed with 20 ml of the equilibrating buffer, and then with 20 ml of an appriopriate eluting compound dissolved in the same buffer, 2-ml fractions were collected and assayed for aldolase activity. The recoveries varied between 86 and 103% with respect to the activity applied. In the control experiment it was demonstrated that native aldolase was not able to bind to the underivatized agarose. All experiments were performed at 5°C. The activity elution profiles were obtained using: (A) 10 mM Mops-NA buffer/0.2 mM EDTA, pH 7.5; (B) 20 mM NaCl (○), 100 mM NaCl (●); (C) 0.25 mM fructose-1,6-P_2; (D) 0.25 mM ATP; (E) 8.5 mM NADH; (F) 20 mM NAD^+ (○), 40 mM NAD^+ (●); (G) 0.25 mM dihydroxyacetone phosphate (DHAP) (○), 0.85 mM DHAP (●); (H) 0.25 mM glyceraldehyde 3-phosphate (GAP) (○), 0.85 mM GAP (●).

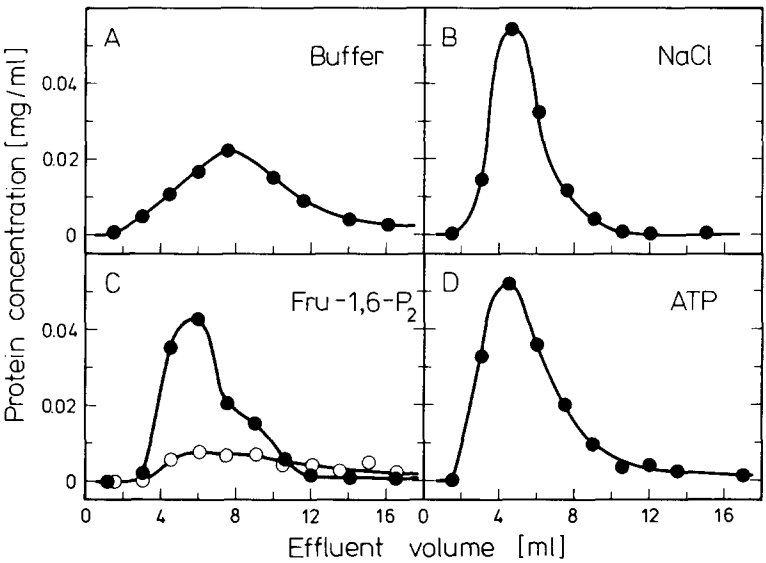

Fig. 2. Chromatography of β-glycerylphosphate derivative of aldolase on an NAD-agarose column. β-Glycerylphosphate derivative of aldolase with the specific activity of 0.12 units/mg protein was prepared according to Lai et al. [14]. The NAD-agarose column was prepared and developed as described in the legend to Fig. 1. Protein concentration was determined using the fluorescamine method [15]. In the control experiment it was shown that native aldolase which had been treated with $NaBH_4$ in the absence of dihydroxyacetone phosphate behaved in the same fashion as untreated enzyme, i.e. it could be sharply eluted with 0.25 mM fructose-1,6-P_2. The protein elution profiles were obtained using: (A) 10 mM Mops-Na buffer/ 0.2 mM EDTA, pH 7.5; (B) 100 mM NaCl; (C) 0.25 mM fructose-1,6-P_2 (○), 1 mM fructose-1,6-P_2 (●); (D) 0.5 mM ATP.

other region of the active center. Therefore, the inactive β-glycerylphosphate derivative of aldolase is able to bind to NAD-agarose. Elution of the modified enzyme requires higher concentration of fructose-1,6-P_2 or ATP compared to the native aldolase, since affinity of these compounds for the second anion binding region is lower [12]. This explains why the effective concentration of any phosphotriose for the elution of the native enzyme is much higher than that which is required for saturation of the specific subsite. Alternatively, it is conceivable that only one binding site is at the active center region; second is outside but in a close proximity to the first one. Both of the above explanations lead to the conclusion that aldolase has two binding sites for NAD^+ and at least one of them is located at the active center. The second site overlaps with the weak binding site for fructose-1,6-P_2 and ATP.

Our results, when compared to those obtained with Cibacron blue F3GA, indicate that the dye and the dinucleotide differ in their interaction with aldolase. The catalytic site of the enzyme is involved in the binding of NAD^+, whereas according to Grazi et al. [9], none of the 3.5 molecules of the dye per aldolase subunit is bound to the active site. However, one of the binding sites for Cibacron blue F3GA is probably identical to the second (weak) site for NAD^+, ATP, and fructose-1,6-P_2. The fact that NAD^+ can be bound specifically to the aldolase molecule might suggest evolutionary relationship between this enzyme and dehydrogenases and kinases. This hypothesis,

however, requires further evidence concerning the specifity of the dinucleotide-aldolase interaction which should be provided using NAD^+ or NADH, rather than empirical probes such as Cibacron blue F3GA.

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