ALLOSTERIC AND ISOSTERIC MODIFIERS OF NADH BINDING TO CYTOPLASMIC MALIC DEHYDROGENASE

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SUMMARY: The binding of NADH to cytoplasmic malic dehydrogenase is shown to be affected by a number of added ligands. One class of ligands appear to be analogs of a substrate for the enzyme, L-malate. These alter the binding constant for NADH without affecting the cooperativity of binding. In contrast, fructose-1,6-diphosphate behaves as an allosteric inhibitor at low enzyme concentrations, apparently by shifting the monomer-dimer equilibrium of the protein to the cooperatively binding dimer. The significance of these results are discussed in terms of a proposed regulatory function for the enzyme.

INTRODUCTION

Malic dehydrogenase is an enzyme normally associated with mitochondrial function, through participation in the tricarboxylic acid cycle. However, eucaryotic cells also have a second, structurally distinct protein, catalyzing the same reaction; this enzyme is localized in the cytoplasm (1-4). In contrast to the mitochondrial enzyme, the role of the cytoplasmic malic dehydrogenase, (S-MDH), has not been clearly defined. Rather, S-MDH has been implicated in a number of metabolic flow schemes, all of which in some way involve the coupling of intra- and extra-mitochondrial metabolism (5-7). Recent studies in our laboratory have demonstrated a cooperative binding of NADH to S-MDH, coupled to a monomer-dimer equilibrium of the protein (8). The existence of such cooperative interactions suggested that the enzyme may have some regulatory functions. Further studies were therefore initiated to search out possible allosteric modifiers of NADH binding. The results presented here show that both allosteric and isosteric modifiers exist. The relationship of these *in vitro* effects to a potential *in vivo* regulatory role for S-MDH is discussed.

MATERIALS AND METHODS

S-MDH was isolated from beef heart and purified by the method of Guha,

Englard and Listowsky (9). NADH and D-malate, A grade, and D,L + allo trisodium isocitrate, C grade, were obtained from Calbiochem. The sodium salts of fructose 1,6-diphosphate (FDP), D-fructose-6-phosphate, D-glucose-6-phosphate, and allo-free D,L-isocitrate were Sigma grade. The barium salt of 3-phosphoglycerate was obtained from Boehringer. Glucose-1,6-diphosphate was a gift from Dr. Grant Bartlett. Measurements of NADH-enzyme interactions were made by following the quenching of protein fluorescence at 340 nm upon excitation at 280 nm. The percent quenching per mole NADH bound was linear with fractional saturation (8). Fluorescence measurements were performed on a Hitachi-Perkin Elmer MPF-IIA spectrophotofluorometer thermostated at 20-21°. All titrations were carried out at pH 6.0 in sodium potassium phosphate buffer, at ionic strength 0.05. The initial volume was 2.0 ml, and all fluorescence measurements were corrected for dilution and concentration dependent non-linearity of the emission. The titrations were performed by equilibrating an enzyme solution for 5 min. in the thermostated cell block, following which 10 µl aliquots of NADH solutions of known concentration were added using an Eppendorf micropipette.

RESULTS

Titrations of S-MDH with NADH were carried out in the presence and absence of added ligand. Two classes of ligands capable of modifying NADH binding were identified. The first class all appear to be analogs of the natural substrate, L-malate. These include D-malate, citrate, and allo-isocitrate. (The effectiveness of allo-isocitrate was inferred from the observation of a change in the NADH binding curve with added D,L + allo material, while added allo-free D,L-isocitrate had no effect). These compounds can all be represented as α- or β-substituted four-carbon dicarboxylic acids, with α-hydroxyl groups of the opposite steric configuration from that of L-malate. Titrations with NADH in the presence of members of this class generated binding curves which showed no increased sigmoid character compared to enzyme alone (Fig. 1). However, these modifiers appear to alter the intrinsic binding constant for NADH, resulting in

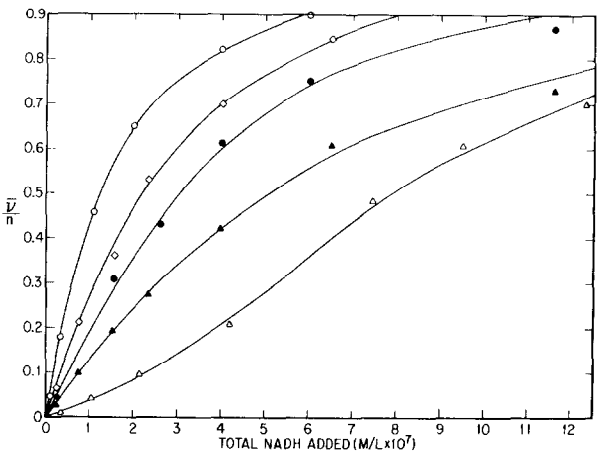

Figure 1. Titration curves of S-MDH with NADH, in the presence and absence of added ligand. The enzyme concentration was 5 µg/ml, and the ligands were added at a concentration of 5×10^{-3}M. (●), enzyme alone; (o),+ D,L, + allo-isocitrate; (◊), + D-malate; (▲), + citrate; (△), + D-fructose-1,6-diphosphate.
The ordinate is fractional saturation of enzyme with respect to NADH. $\bar{\nu}$ = moles NADH bound per mole enzyme, and n = NADH binding sites per mole enzyme.

a binding curve which is displaced relative to enzyme alone. Titrations at protein concentrations in the range 10-200 µg/ml indicated that the protein concentration dependence reported previously (8), was unaltered in the presence of these ligands.

The second class of effectors has as yet only one member, D-fructose-1, 6-diphosphate (FDP). At low enzyme concentrations the binding curve for NADH became distinctly sigmoidal in the presence of FDP (Fig. 1). Other phosphorylated intermediates of glycolysis, such as glucose-6-phosphate, glucose-1,6-diphosphate,fructose-6-phosphate, and 3-phosphoglycerate, showed no effect. An examination of the Scatchard plots obtained at different enzyme concentrations showed that in the presence of FDP the binding is characteristic of high concentrations of enzyme alone (Fig. 2), indicating the FDP may act by displacing the monomer-dimer equilibrium to favor formation of the cooperative dimer (8).

The two classes can be further distinguished by following the changes in NADH binding obtained upon titration with the appropriate effector (Fig. 3). The addition of citrate produced a small inhibition in a manner reflecting a

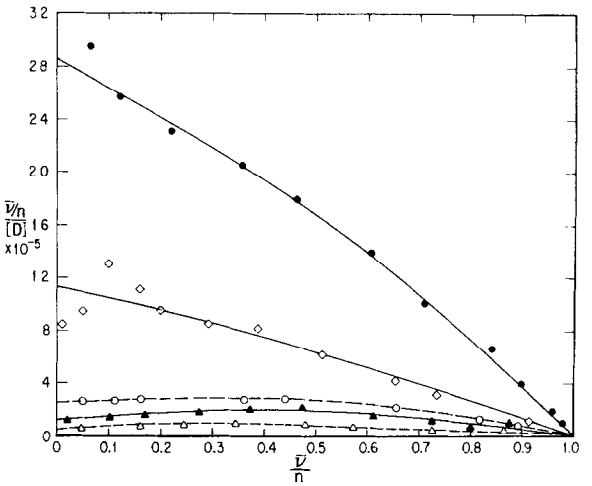

Figure 2. Scatchard plots of S-MDH titrations with NADH at different enzyme concentrations, in the presence and absence of FDP. The solid lines are titrations of enzyme alone. (●), 7 µg/ml enzyme; (◊), 70 µg/ml enzyme; (▲), 210 µg/ml enzyme. The broken lines are titrations in the presence of 10^{-2}M FDP. (o), 7 µg/ml enzyme; (Δ), 70 µg/ml enzyme.

The abscissa is fractional saturation, and the ordinate is fractional saturation divided by the free NADH concentration.

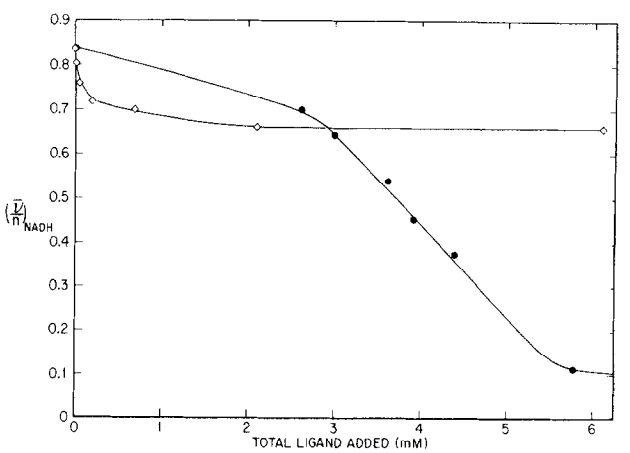

Figure 3. Inhibition of NADH binding by added citrate and FDP. The enzyme concentration was 5 µg/ml, and the NADH concentration was 1.2×10^{-6}M. (◊), citrate titration; (●), FDP titration. The ordinate is fractional saturation of enzyme with respect to NADH.

normal binding isotherm. In contrast, the inhibition of NADH binding by FDP showed a marked cooperativity. The concentration for half-maximal inhibition was approximately $1x10^{-5}$M for citrate, and $4x10^{-3}$M for FDP.

DISCUSSION

Many of the recent advances in the understanding of metabolic control mechanisms have followed on the identification of regulatory enzymes and the nature of their controls. Common indicators for the possible existance of regulatory properties are the position of the enzyme at some branch point in a metabolic pathway, and the effective irreversibility of the enzymatic reaction catalyzed (10). Since S-MDH did not appear to satisfy either of those conditions, there was no reason to suspect that it functioned in any regulatory capacity. However, the observation that S-MDH can bind NADH in a cooperative manner, together with the specific and striking allosteric properties exhibited by FDP, required a re-evaluation of the possible significance of the enzyme in metabolic regulation.

The existence of FDP as an allosteric effector strongly suggests the involvement of S-MDH in phenomena related to the control of glycolysis. It has been generally accepted that a major control point in glycolysis is located at the phosphofructokinase reaction (PFK)(11-13). The interplay between activators and inhibitors of this enzyme is such as to stimulate PFK, and thus accelerate glycolysis, under anaerobic conditions, while the enzyme is inhibited and glycolysis limited under aerobic conditions. Parallel studies have been carried out on *in vivo* levels of glycolytic intermediates in rat brain (14) and heart (15). These demonstrated that the steady-state concentration of FDP increased 2 to 5-fold in going from aerobic to anerobic conditions. The high degree of cooperativity shown by the inhibition of NADH binding to S-MDH as a function of added FDP (Fig. 3), allows the FDP effect to operate over a concentration span consistent with that observed *in vivo*. However, the concentration of FDP required for half-maximal inhibition is approximately one order of magnitude larger than that found *in vivo*. This discrepancy may be due to several factors. Observations

with a number of preparations of enzyme indicate that the response to FDP is very labile. This lability of an allosteric effect is a characteristic shared by many allosteric enzymes. As a result, the effectiveness of the FDP response may be considerably altered in the purified preparation, relative to the enzyme in the native state. In addition, preliminary kinetic studies (Vetterlein and Cassman, in progress), indicate that oxaloacetate, the co-substrate for the reaction with NADH, can induce cooperative binding of NADH. The possibility that oxaloacetate may modify FDP binding as well, is currently being investigated.

The results presented suggest that S-MDH may play a role in regulating the flow of cytoplasmic NADH. Newsholme has suggested that the control of lactic dehydrogenase in red muscle and the mechanism for the re-oxidation of glycolytic NADH may be related (16). Such a link may be provided by the controls on S-MDH. A possible model would involve a competition between S-MDH and lactic dehydrogenase for cytoplasmic NADH. Under anaerobic conditions, the increased levels of FDP would inhibit binding of NADH to S-MDH and allow a reoxidation of cytoplasmic NADH through the lactic dehydrogenase system. Under aerobic conditions, the lowered levels of FDP would relax the inhibition on NADH binding, and thus funnel reducing equivalents into the mitchondria *via* S-MDH and the malate shuttle. S-MDH would therefore function as a secondary control point, by responding to the level of phosphofructokinase activity.

The cooperative interactions observed with FDP are characteristic of an allosteric modifier, i.e., a modifier which binds at a site distinct from the active site. In contrast, citrate, *allo*-isocitrate, and *D*-malate neither affect the degree of cooperativity of NADH binding, nor its dependence on protein concentration. Since these latter compounds can all be represented as *L*-malate analogs, it seems most probable that they act by binding at the same site as the substrate, and alter the affinity for NADH through local electrostatic or steric interactions. Of this group, only citrate is normally found in cells, and its effect on NADH binding appears too small to be significant. Thus, there are no

strong reasons to believe that such isosteric effectors are of physiological importance.

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