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1-ANILINO-8-NAPHTHALENE SULFONATE AS A COENZYME-COMPETITIVE INHIBITOR OF YEAST GLYCERALDEHYDE-3-PHOSPHATE DEHYDROGENASE: MULTIPLE INHIBITION STUDIES

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SUMMARY

1-Anilino-8-naphthalene sulfonate (ANS) inhibits dehydrogenase activity of yeast glyceraldehyde-3-phosphate dehydrogenase (D-glyceraldehyde-3-phosphate: NAD^+ oxidoreductase (phosphorylating), EC 1.2.1.12). Inhibition is noncompetitive with respect to substrate, glyceraldehyde 3-phosphate, and strictly competitive with inorganic phosphate and NAD^+. Multiple inhibition studies using ANS and several adenine derivatives, known to function competitively with respect to NAD^+, were carried out. A graphical method was applied to determine the extent to which interactions between inhibitor pairs ANS–adenosine, ANS–adenosine 5'-monophosphate (AMP) and ANS–adenosine diphosphate (ADP) occur on the enzyme. A slight negative interaction was found between ANS and adenosine, the interaction constant α being 1.26, while for inhibitor pair ANS–AMP α was found to be 1.8, suggesting some increase in interaction. No difference was revealed between AMP and ADP effects. The results are consistent with the binding of ANS in a region distinct from the site interacting with the adenosine portion of the NAD^+ molecule. Some positively charged group on the enzyme is supposed to be located in the vicinity of the dye binding site.

INTRODUCTION

Multiple inhibition studies using various combinations of coenzyme-competitive inhibitors, capable of binding at different sites on the enzyme molecule, have been very useful for elucidating the nature of the active center of some dehydrogenases[1–6]. Glyceraldehyde-3-phosphate dehydrogenase (D-glyceraldehyde-3-phosphate:NAD^+ oxidoreductase (phosphorylating), EC 1.2.1.12) from baker's yeast was recently shown to be competitively inhibited by several adenine derivatives, presumably due to their structural analogy to adenine portion of the coenzyme molecule[7,8]. On the other hand, we have found, that 1-anilino-8-napthalene sulfonate (ANS), structurally unrelated

Abbreviation: ANS, 1-anilino-8-naphthalene sulfonate.

to NAD^+, is a rather efficient coenzyme-competitive inhibitor of the dehydrogenase, inhibitor constant K_i being 0.055 mM[9]. The binding of ANS involves interaction with a relatively nonpolar region of the enzyme molecule, as evidenced by fluorescence measurements[9]. Strictly coenzyme-competitive character of inhibition observed with ANS suggests the possibility of some contribution of dispersion forces to the binding of NAD^+, since dye and coenzyme binding sites overlap.

In order to evaluate, which portion of the coenzyme molecule can have access to the hydrophobic region of the active site, multiple inhibition studies were undertaken. Various combinations of ANS and adenine derivatives were used as inhibitor pairs, and multiple inhibition was analyzed by applying the graphical method of Yonetani and Theorell[2]. The interaction of coenzyme-competitive inhibitors with glyceraldehyde-3-phosphate dehydrogenase was also studied by means of fluorescence technique.

MATERIALS AND METHODS

Crystalline glyceraldehyde-3-phosphate dehydrogenase was prepared from baker's yeast according to the method of Krebs[10] with slight modifications. $(NH_4)_2SO_4$ was removed from the enzyme solution by passing it through a column of Sephadex G-50, equilibrated with 5 mM EDTA, pH 7.5. The $A_{280\ nm}:A_{260\ nm}$ ratio of the enzyme preparations varied between 1.78 and 1.85. NAD^+, adenosine and ADP were obtained from Reanal, DL-glyceraldehyde 3-phosphate (barium salt of diethyl acetal) was the product of Calbiochem, AMP was obtained from Fluka AG. The ammonium salt of ANS was purified by several recrystallizations from hot water after filtration of the hot solution through activated charcoal. The pale yellow-green crystals had a molar extinction coefficient of $5.6 \cdot 10^3$ at 350 nm in 0.1 M phosphate buffer, pH 6.8. The concentrations of ANS, as well as of adenine derivatives, were determined spectrophotometrically. The concentrations of NAD^+ and glyceraldehyde 3-phosphate were determined enzymatically using glyceraldehyde-3-phosphate dehydrogenase: the increase in NADH in the presence of limiting amounts of these reagents was followed up.

Glyceraldehyde-3-phosphate dehydrogenase catalyzed reduction of NAD^+ was studied at 25 °C in 3-ml reaction mixtures containing 0.1 M sodium glycine buffer, pH 8.2, 5 mM EDTA, 5 mM sodium arsenate. The concentrations of the other components of the reaction mixture (NAD^+, substrate, inhibitors and enzyme) will be included in specific descriptions of individual experiments. In the experiments, described in Fig. 2, sodium arsenate was omitted. Reactions were initiated by adding glyceraldehyde 3-phosphate to the reaction mixture, in which the enzyme had been equilibrated for 1 min. In all inhibition studies the inhibitor was included in the equilibration process. The reaction was followed by the increase in absorbance at 340 nm in a SF-4 spectrophotometer equipped with a device for kinetic experiments, using a 0.1 absorbance full scale. The initial reaction rates in the presence and absence of inhibitors were plotted according to Lineweaver and Burk[11] or Yonetani and Theorell[2].

Fluorescence emission measurements were carried out at room temperature (20 °C) in a spectrophotometric attachment G-3 for a Hitachi Spectrophotometer EPS-3; cells of 1-cm light-path were used. The excitation and emission wave lengths

were 350 and 490 nm, respectively. All fluorescence measurements were corrected for fluorescence of dye, enzyme and other reagents when added.

RESULTS AND DISCUSSION

The ANS-induced inhibition of dehydrogenase activity of yeast glyceraldehyde-3-phosphate dehydrogenase studied as a function of varying substrate concentrations, was observed to be noncompetitive with respect to glyceraldehyde 3-phosphate, as shown in Fig. 1. Similar studies with inorganic phosphate as the variable demonstrated strictly competitive relationship to exist between ANS and phosphate (Fig. 2). The results obtained with arsenate were essentially the same. An important role of ionic interactions between ANS and the enzyme follows from these results, the dis-

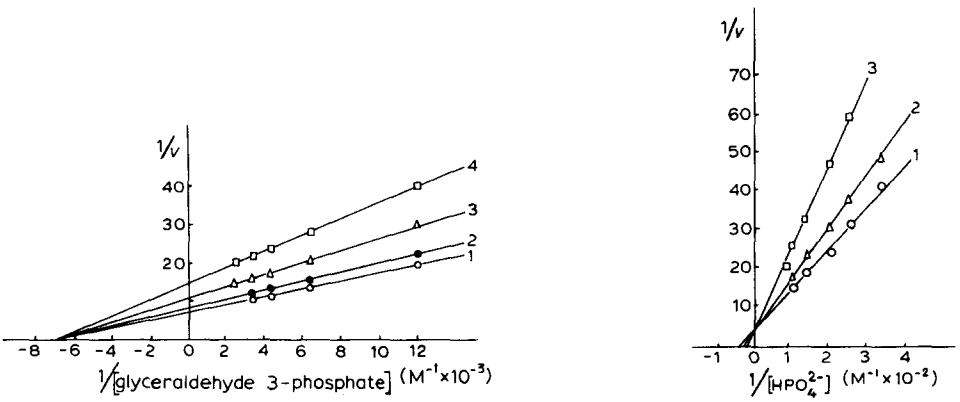


Fig. 1. Inhibition of yeast glyceraldehyde-3-phosphate dehydrogenase activity by ANS with respect to varying glyceraldehyde 3-phosphate concentrations. Reaction mixtures contained 0.1 mM NAD^+, $2.4 \cdot 10^{-8}$ M enzyme. The concentration of glyceraldehyde 3-phosphate varied from 0.077 to 0.388 mM. The concentrations of ANS used were as follows: Line 1, zero; Lines 2, 3 and 4, 0.07 mM, 0.14 mM and 0.21 mM, respectively. The v values in Figs 1–3 are given in arbitrary units.

Fig. 2. Inhibition of yeast glyceraldehyde-3-phosphate dehydrogenase activity by ANS with respect to varying inorganic phosphate concentrations. Reaction mixtures contained 0.1 mM NAD^+, 0.3 mM glyceraldehyde 3-phosphate, $1.28 \cdot 10^{-8}$ M enzyme. The concentration of potassium phosphate varied from 3 to 12.5 mM. The concentrations of ANS used were as follows: Line 1, zero; Lines 2 and 3, 0.08 mM and 0.16 mM, respectively.

placement of the dye from its complex with the enzyme by polyvalent anions being due to a competition for a common positively charged group on the protein surface. It may be assumed therefore, that ANS binding site on the protein is formed from a combination of a nonpolar region and some positively charged group situated in its close proximity. The $-SO_3^-$ group of ANS, phosphate and arsenate anions could then prevent one another from binding at this site.

Noncompetitive character of the dehydrogenase inhibition by ANS with respect to glyceraldehyde 3-phosphate (Fig. 1) indicates that the substrate and ANS bind at different sites. It is of interest in this connection, that ionic interactions are of importance as a contributory factor also in the binding of glyceraldehyde 3-phosphate to the enzyme. Purely competitive character of inorganic phosphate inhibition of

glyceraldehyde-3-phosphate dehydrogenase with respect to substrate[12,13] may be interpreted to be the result of the competition for a common positively charged group of the protein between inorganic phosphate anion on the one hand and the anionic group of glyceraldehyde 3-phosphate on the other hand.

Presence of some cationic site in the vicinity of the essential cysteine residue in the active center of glyceraldehyde-3-phosphate dehydrogenase was suggested by several authors[14,15]. This site is, however, inaccessible for ANS, since the dye does not interfere with the interaction of the substrate and the enzyme. The hydrophilic character of the amino acid residues neighbouring the essential cysteine in the primary structure of glyceraldehyde-3-phosphate dehydrogenase[16] may partly account for a restricted access of the dye to this region of the active center.

On the other hand, as seen from Fig. 3, ANS is strictly competitive with NAD^+,

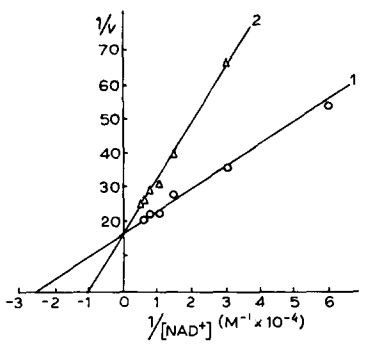


Fig. 3. Inhibition of yeast glyceraldehyde-3-phosphate dehydrogenase activity by ANS with respect to varying NAD^+ concentrations. Reaction mixtures contained 0.33 mM glyceraldehyde 3-phosphate, $1.28 \cdot 10^{-8}$ M enzyme. The concentration of NAD^+ varied from 0.016 to 0.198 mM. Line 1, no ANS; Line 2, 0.083 mM ANS.

the inhibitor constant K_i being 0.055 mM. This value has been found to be in good agreement with the fluorescence titration data on the dissociation constant of the enzyme–ANS complex[9]. Binding of such a complex molecule as NAD^+ to a protein is expected to involve contributory interactions arising from different portions of the coenzyme. The importance of the adenine moiety of NAD^+ molecule for the binding to yeast glyceraldehyde-3-phosphate dehydrogenase was established by demonstrating the strictly coenzyme-competitive character of adenine derivatives as inhibitors[7,8]. Purely coenzyme-competitive nature of the binding of both ANS and adenine derivatives suggests the binding of these compounds at the NAD^+ binding site of the enzyme. The interactions of the different types of inhibitors with the protein are expected to occur at different regions of the coenzyme-binding site.

The possibility exists, therefore, that ANS and adenine derivatives could be simultaneously bound to the dehydrogenase. Such a possibility was investigated by multiple inhibition analysis. For this aim simultaneous action of various pairs of coenzyme-competitive inhibitors was studied, the data obtained being plotted according to Yonetani and Theorell[2]. Initial rates were measured at four different concentrations of ANS with a second inhibitor present at a constant concentration. This series of four reactions was then repeated so that a total of three or four different

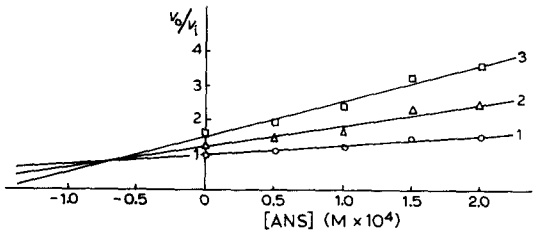
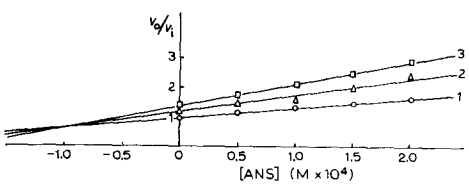


Fig. 4. Multiple inhibition of yeast glyceraldehyde-3-phosphate dehydrogenase by ANS and adenosine. Reaction mixtures contained 0.1 mM NAD^+, 0.33 mM glyceraldehyde 3-phosphate, $1.6 \cdot 10^{-8}$ M enzyme. The concentration of ANS varied from 0.05 to 0.2 mM. Line 1, no adenosine; Lines 2 and 3, 1.4 mM and 2.3 mM adenosine, respectively.

concentrations of the second inhibitor was employed. The data obtained were plotted as the ratio of the initial velocity in the absence of inhibitor (v_0) to the initial velocity in the presence of inhibitor (v_i) against the concentration of this inhibitor.

The multiple inhibition observed with inhibitor pair ANS–adenosine, plotted in this manner, resulted in a converging line relationship (Fig. 4). The interaction constant, α, calculated from this plot[2] equaled 1.26. The coenzyme-competitive inhibitor constant of ANS, K_i, was calculated from the slopes of Lineweaver–Burk plots[11] of the data obtained under the conditions identical to those used in the multiple inhibition studies. Figs. 5 and 6 show the results obtained by using the



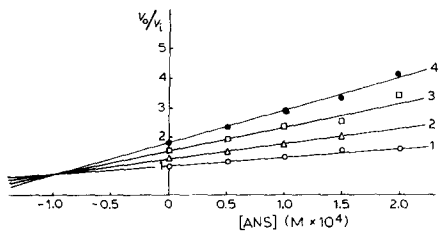


Fig. 5. Multiple inhibition of yeast glyceraldehyde-3-phosphate dehydrogenase by ANS and AMP. Conditions as in Fig. 4. Line 1, no AMP; Lines 2 and 3, 0.16 mM and 0.4 mM, respectively.

Fig. 6. Multiple inhibition of yeast glyceraldehyde-3-phosphate dehydrogenase by ANS and ADP. Conditions as in Fig. 4. Line 1, no ADP; Lines 2, 3 and 4, 0.165 mM, 0.33 mM and 0.66 mM ADP, respectively.

ANS–AMP and ANS–ADP inhibitor pairs. The multiple inhibition studies again resulted in a series of converging lines, the interaction constant, α, being equal to 1.8 in both cases. It should be noted that non-linear relationships in the plot of v_0/v_i against ANS were observed when the inhibitory effect of ANS used in concentrations higher than 0.1 mM was measured at sufficiently high AMP concentrations (0.6 mM and more), some conformational changes of the protein probably being responsible for this effect.

The results obtained in the present work are consistent with ANS and adenine derivatives being bound at different sites of the active center of yeast glyceraldehyde-3-phosphate dehydrogenase, since in the case of their mutual exclusion a series of parallel lines would have been obtained[2]. The interaction of the components of the

inhibitor pairs with the enzyme is, however, not strictly independent, the α value being more than unity. Interaction constant, $\alpha = 1.26$, indicates a slight negative interaction to occur between ANS and adenosine, simultaneously bound to the enzyme. Substitution of AMP for adenosine results in a slight increase in mutual repulsion of the inhibitors ($\alpha = 1.8$), whereas no difference was found between AMP and ADP effects.

The interaction of ANS and other ligands with glyceraldehyde-3-phosphate dehydrogenase was further studied by means of fluorescence technique. ANS binding to the enzyme is accompanied by a marked enhancement of fluorescence of the dye and a shift of the emission maximum from about 515 nm to 490 nm. As shown in Fig. 7, the enzyme-induced fluorescence of ANS is progressively lowered upon

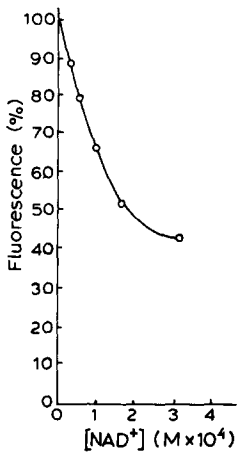


Fig. 7. Effect of NAD^+ on enzymes–ANS fluorescence. The assay solution contained 1.64 μM enzyme and 17.5 μM ANS in 3 ml of 100 mM glycine–NaOH buffer, pH 8.5. The fluorescence intensity at 490 nm (excitation at 350 nm) was determined before and after the additions of NAD^+ (10 mM). Fluorescence is expressed in arbitrary units.

addition of NAD^+. Adenosine and AMP in concentrations up to 6.6 mM had no effect on the fluorescence intensity of the ANS–enzyme complex (7.2 μM enzyme, 52 μM ANS), whereas 0.5 mM ADP lowered the fluorescence 10%. P_i decreased the emission 10% at 6.9 mM concentration and 16% at 20 mM, suggesting some contribution of ionic bonding to the dye–protein association.

It seems probable from these results, that adenosine or AMP may bind to the enzyme simultaneously with ANS, with no measurable interaction, whereas ADP by virtue of its larger size and charge extends closer to the dye-binding site with a concomitant quenching of fluorescence. The decrease of ANS–dehydrogenase fluorescence upon addition of coenzyme may indicate a displacement of the dye from the complex due to a competition for a common site, the results being in line with the conclusions following from the kinetic experiments.

Another possibility, however, is not excluded: that fluorescence quenching of enzyme-bound ANS in the presence of NAD^+ reflects some conformational changes induced in the protein by the binding of coenzyme. Further investigation is needed to elucidate this problem.

REFERENCES

1 T. Yonetani, *Biochem. Z.*, 338 (1963) 300.
2 T. Yonetani and H. Theorell, *Arch. Biochem. Biophys.*, 106 (1964) 243.
3 B. M. Anderson and M. L. Reynolds, *Arch. Biochem. Biophys.*, 111 (1965) 1.
4 M. L. Fonda and B. M. Anderson, *Arch. Biochem. Biophys.*, 120 (1967) 49.
5 Soo Ja Kim and B. M. Anderson, *J. Biol. Chem.*, 243 (1968) 3351.
6 A. McPherson, Jr, *J. Mol. Biol.*, 51 (1970) 39.
7 N. K. Nagradova, M. K. Vorona and R. A. Asryants, *Biokhimiya*, 34 (1969) 627.
8 Shih Tzy Yang and W. C. Deal, Jr, *Biochemistry*, 8 (1969) 2806.
9 N. K. Nagradova, R. A. Asryants and M. V. Ivanov, *Experientia*, 27 (1971) 1169.
10 E. G. Krebs, in S. P. Colowick and N. O. Kaplan, *Methods in Enzymology*, Vol. I, Academic Press, New York, 1955, p. 407.
11 H. Lineweaver and D. Burk, *J .Am. Chem. Soc.*, 56 (1934) 658.
12 S. E. Severin and N. K. Nagradova, *Dokl. Akad. Nauk. SSSR*, 121 (1958) 519.
13 T. Keleti and M. Telegdi, *Acta Physiol. Acad. Sci. Hung.*, 16 (1959) 235.
14 A. Fenselau, *J. Biol. Chem.*, 245 (1970) 1239.
15 E. Cseke and L. Boross, *Acta Biochim. Biophys. Acad. Sci. Hung.*, 5 (1970) 385.
16 R. N. Perham and J. I. Harris, *J. Mol. Biol.*, 7 (1963) 316.