BINDING OF DIHYDRONICOTINAMIDE FORMYCIN DINUCLEOTIDE TO NAD-SPECIFIC ISOCITRATE DEHYDROGENASE FROM BOVINE HEART

Shan S. WONG

The Biochemistry Program, Department of Chemistry, University of Lowell, Lowell, MA 01854, USA

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1. Introduction

NAD-specific isocitrate dehydrogenase (EC 1.1.1.41) from bovine and porcine heart is an allosteric enzyme consisting of 8 subunits with nearly identical molecular weights [1]. Recent studies suggest that these subunits are composed of 2 [2] or 4 [3] different polypeptide chains. While ADP is a positive modifier and has the effect of aggregating the enzyme into higher molecular forms [4], reduced pyridine nucleotides inhibit the catalytic activity [5,6]. It has been shown [7] that the enzyme in its octameric form has 4 NADH binding sites. The fluorescence of NADH was enhanced 20-fold on binding to one of these sites.

Although the structural requirements for NAD^+ cosubstrate and ADP activator have been investigated in detail by using various analogues [8], the NADH inhibitor binding sites have not been examined. We report here the interaction of dihydronicotinamide formycin dinucleotide (NFDH) with the enzyme from bovine heart. This NADH analogue contains, instead of adenosine, a formycin portion which is fluorescent. Thus NFDH is a double fluorophor, containing two fluorescent moieties, the 7-aminopyrazolopyrimidine and dihydronicotinamide bases (fig.1). Its fluorescence behavior in the presence of NAD-specific isocitrate dehydrogenase is reported.

2. Materials and methods

NAD-specific isocitrate dehydrogenase was isolated from bovine heart [1] and had spec. act. 28 units/mg protein as assayed under the standard conditions in [9]. The concentration of the purified enzyme was estimated from the extinction coefficient E_{278} (0.1%) = 0.53 cm^{-1} [9] and mol. wt 320 000 [1]. Yeast alcohol dehydrogenase was obtained from Worthington Biochemical Corp. and snake venom phosphodiesterase from Boehringer-Mannheim.

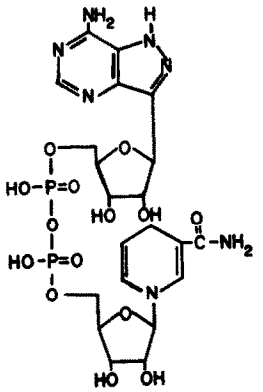

Fig.1. NFDH = dihydronicotinamide formycin dinucleotide

Formycin was purchased from Meiji Seika Kaisha Ltd., Tokyo. Nicotinamide formycin dinucleotide (NFD^+) was synthesized according to [10]. NFDH was prepared by the reduction of NFD^+ in the presence of yeast alcohol dehydrogenase and purified on a column of DEAE-cellulose with a 0–0.3 M ammonium acetate gradient (pH 8.5) [11].

All spectrophotometric and fluorometric measurements were carried out in 0.17 M sodium *N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonate (Na-Hepes) (pH 7.2), 25°C. NFDH inhibition kinetics were measured in 0.2 ml final vol. containing 1.1 mM manganese isocitrate, 0.17 M Na-Hepes (pH 7.2) varying concentrations of NAD^+ and fixed amounts of NFDH and isocitrate dehydrogenase. The rate of A_{340} increase was monitored on a Cary 14 spectrophotometer.

3. Results and discussion

3.1. *Spectral characteristics of NFDH*

The ultraviolet absorption spectrum of NFDH shows characteristics of both the formycin moiety with a maximum at 295 nm and shoulders at 305 and 320 nm [12] and the dihydronicotinamide moiety with a maximum ~340 nm (fig.2A). Fluorescence spectrum of the compound excited at 290 nm reveals the formycin emission band at 340 nm and the reduced nicotinamide band at ~460 nm. The latter emission may be due to both direct absorption of light by the dihydronicotinamide base and energy transfer from 7-aminopyrazolopyrimidine base. That energy is transferred between the two bases is evident from the excitation spectrum monitored at 460 nm. When the molecule was hydrolyzed by snake venom phosphodiesterase, both the 295 nm shoulder of the excitation spectrum (monitored at 460 nm) and the emission band at 460 nm (excited at 290 nm)

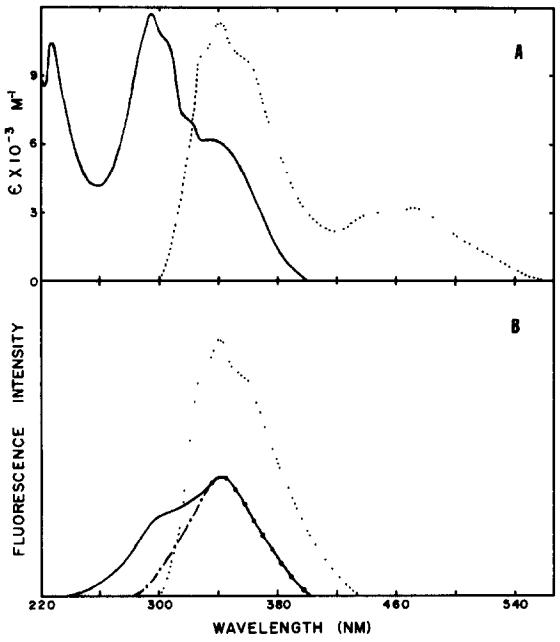

Fig.2. (A) Absorption and fluorescence spectra of NFDH. The absorption spectrum (——) was measured in 0.17 M Na-Hepes (pH 7.2) at 25°C. The emission spectrum (. . .) was measured in the same buffer excited at 290 nm. (B) Fluorescence spectrum of NFDH after treatment with snake venom phosphodiesterase. The excitation spectra were monitored at 460 nm before (——) and after (–·–) treatment. The emission spectrum (. . .) was excited at 290 nm after treatment.

disappeared (fig.2B). Since the excited state lifetime of formycin is <1 ns [12], the energy transfer between the two bases probably indicates that the molecule exists to a certain extent in the stacked conformation similar to that of NAD^+ and NADH [13].

3.2. *Inhibition of isocitrate dehydrogenase*

Since NFD^+ is a cosubstrate for isocitrate dehydrogenase [14], NFDH would be expected to function as an allosteric inhibitor. Kinetic analysis revealed that NFDH was a competitive inhibitor against NAD^+ similar to NADH [15]. The inhibition constant of NFDH was found to be 31 μM compared to that of 40 μM for NADH obtained earlier [15]. Whereas the K_m for NFD^+ is ~2–3-times greater than that for NAD^+, the structural differences between formycin and adenosine do not seem to affect the binding of the inhibitor to the allosteric site. That the detailed structures of the active site and the inhibitory allosteric site are different is further supported by the inhibitory effect of dihydronicotinamide hypoxanthine dinucleotide (deamino-NADH). While the replacement of adenine portion of NAD^+ by hypoxanthine led to loss of its cosubstrate activity [15], deamino-NADH is an inhibitor with a K_d of 0.21 mM.

3.3. *Titration of isocitrate dehydrogenase with NFDH*

As with NADH [7], the fluorescence intensity of NFDH (excited at 340 nm) is enhanced >20-fold and the emission maximum shifted from 460–430 nm in the presence of NAD-specific isocitrate dehydrogenase. This is not surprising since both molecules contain the dihydronicotinamide base. NFDH, however, contains an additional fluorophor, 7-aminopyrazolopyrimidine. It would be of interest to learn the binding environment of the adenine ring of NADH by studying the fluorescence behavior of this particular fluorophor. Unfortunately, its excitation maximum at 295 nm coincided with that of the aromatic residues of the protein, making it very difficult to isolate the fluorescence emitted from the base. However, when excited at 310 nm, where the protein absorption is minimal, little fluorescence was observed at 340 nm. This may indicate that its binding site is relatively less hydrophobic than that for the dihydronicotinamide ring. In 95% ethanol, the emission from NFDH at 340 nm is enhanced >3-fold.

The large fluorescence enhancement of NFDH at 430 nm in the presence of the enzyme has enabled

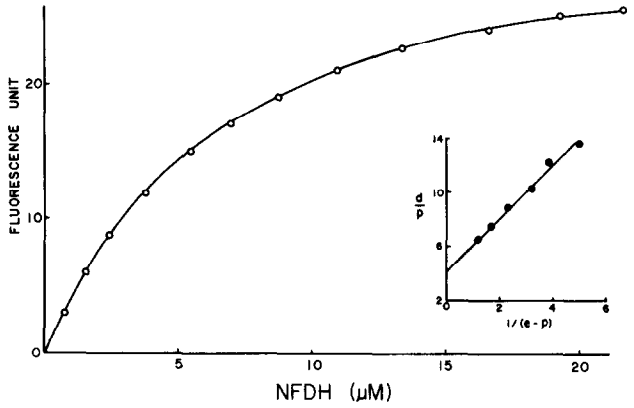

Fig.3. NFDH fluorescence titration of NAD-specific isocitrate dehydrogenase. Enzyme was 1.0 μM in 0.17 M Na-Hepes (pH 7.2). The solution was excited at 340 nm and fluorescence intensity measured at 430 nm after each addition of NFDH. Insert: Stockell plot of titration data.

us to study the K_d and the number of NFDH binding sites under various conditions. Fig.3 shows a fluorescence titration curve of a fixed concentration of NAD-specific isocitrate dehydrogenase with successive additions of NFDH. The data were analyzed by the equation of Stockell [16]:

$$\frac{d}{p} = \frac{K_d}{(e-p)} + n$$

where d is the total concentration of NFDH, e the total concentration of protein, p the concentration of NFDH bound, K_d the dissociation constant and n the number of NFDH binding sites.

The results of the analysis are listed in table 1. In the absence of any added substrate or ADP, four binding sites were detected with an overall K_d of 2.0 μM. This is in agreement with data obtained by gel filtration [7]. Fluorescence titration with NADH, however, revealed only one binding site [7]. The ability of NFDH to detect all 4 sites may be due to the characteristic absorption band of formycin. The presence of the allosteric effector ADP did not affect the number of binding sites. However, the K_d was slightly increased, reflecting a possible change of conformation induced by ADP.

In the presence of saturating manganese isocitrate, n was increased to 8. The additional binding sites detected are probably those of the active sites due to

Table 1
NFDH binding to isocitrate dehydrogenase as revealed by fluorescence titration[a]

Substances added	n[b]	K_d (μM)b
None	4.1	2.0
ADP (1.0 mM)	4.1	4.7
MI (1.0 mM)[c]	7.6	3.8
NAD^+ (0.57 mM)	1.4	4.4

[a] The data were obtained from titration curves as shown in fig.3

[b] K_d and n were obtained from the slope and intercept, respectively, of a Stockell plot

[c] The concentration of total manganese and D,L-isocitrate were 1.3 and 5.3 mM, respectively. The concentration of manganous D,L-isocitrate (MI) was calculated to be 1.0 mM by using the stability constant $K = 1.056\ mM^{-1}$ of [17]

the formation of enzyme–substrate–product ternary complex. The number of active sites/octamer of enzyme has not been reported. If the observation obtained here is such that all the NFDH binding sites are saturated, the enzyme would have 4 active sites/octamer.

NAD^+ has been reported not to affect the binding site of NADH detected by fluorescence, but partially displace NADH bound to the enzyme revealed by ultrafiltration [7]. As shown in table 1, NAD^+ also affected the binding of NFDH. In the presence of 0.57 mM NAD^+, the number of NFDH binding sites detected was reduced to one which probably corresponded to that detected by NADH fluorescence. It thus seems that the 4 NFDH binding sites are heterogeneous, as suggested in [7].

Although nicotinamide 1,N^6-ethenoadenine dinucleotide (ϵNAD^+) is not a cosubstrate for NAD-specific isocitrate dehydrogenase [14]; ϵNADH may inhibit the enzyme by binding to the allosteric regulatory sites as in the case for deamino-NADH mentioned earlier. If this is the case, it would be of interest to compare its behavior with that observed here. Such experiments are currently under investigation.

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