Nicotinamide Adenine Dinucleotide Binding and Promotion of Enzyme Activity: Model Based on Affinity Labeling of 3α,20β-Hydroxysteroid Dehydrogenase with a Nucleoside†

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ABSTRACT: 5′-[*p*-(Fluorosulfonyl)benzoyl]adenosine (FSA) was used to affinity-label the NADH binding region of 3α,20β-hydroxysteroid dehydrogenase (3α,20β-HSD) to further test our hypothesis [Sweet, F., & Samant, B. R. (1980) *Biochemistry 19*, 978–986] that 3α and 20β activities occur at the same active site. Incubation of 3α,20β-HSD (0.45 μM) with FSA (125 μM) at pH 7.0 and 0 °C caused simultaneous loss of 3α and 20β activities by a first-order kinetic process, with $t_{1/2}$ = 300 min for both activities. Dinucleotides and adenosine mononucleotides which acted as competitive inhibitors protected 3α,20β-HSD against inactivation by FSA in a concentration-dependent manner, in the order reduced nicotinamide dinucleotide phosphate > oxidized nicotinamide dinucleotide phosphate > adenosine diphosphate-ribose > adenosine diphosphate > adenosine monophosphate (AMP) > adenosine. Oxidized and reduced nicotinamide mononucleotides (NMH and NMNH) and steroid substrates did not protect 3α,20β-HSD against affinity labeling by FSA. Although NMN was not a competitive inhibitor of 3α,20β-HSD, NMN with AMP and also AMP with NMNH produced positive cooperativity for competitive inhibition of 3α,20β-HSD. The results from FSA affinity labeling of the cofactor region confirm that both 3α and 20β activities share the same active site of 3α,20β-HSD and suggest a model of cofactor binding and promotion of enzyme activity. The adenosine 5′-phosphate component anchors the NAD or NADH to an adenosine domain in the cofactor binding region. The nicotinamide nucleotide component then carries out the hydrogen-transfer reaction at a neighboring domain near the steroid binding region.

We have previously conducted experiments with affinity-alkylating substrate analogues of 3α,20β-hydroxysteroid dehydrogenase (3α,20β-HSD)[1] and found that both 3α and 20β enzyme activities share a common site (Sweet & Samant, 1980a,b; Sweet et al., 1980). Others recently verified our findings (Strickler et al., 1980). To further test our hypothesis that the two activities occur at the same active site, we wished to use a nucleoside affinity label for the NADH cofactor binding region.

The nucleoside affinity label 5′-[*p*-(fluorosulfonyl)-benzoyl]adenosine (FSA) was used in the present work to study the NADH cofactor binding region of 3α,20β-HSD. FSA was known to selectively react with amino acids in the ATP, NADH, or NADPH binding regions at the active sites of a variety of enzymes (Esch & Allison, 1978a,b; Pal et al., 1975; Wyatt & Colman, 1977; Zoller & Taylor, 1979; Likos et al., 1980). Our present experiments with FSA were designed to provide further insight into the nature of 3α and 20β activities with respect to cofactor binding at the active site of 3α,20β-HSD. The adenosine and nicotinamide components of NADH were tested for binding to the cofactor region to explore the mechanism by which NAD and NADH promote 3α and 20β enzyme activities. This study may be of general importance because NAD and NADH binding and promotion of enzyme activity are essential for many biochemical processes in all plants and animals.

Experimental Procedures

Materials

Progesterone, cortisone, and 17β-hydroxy-5α-androstan-3-one were purchased from Steraloids, Inc., Wilton, NH. Nucleotides NAD, NADH, adenosine, AMP, ADP, nicotinamide mononucleotide in oxidized (NMN) and reduced (NMNH) forms, and adenosine 5′-diphosphate-ribose (ADPR) were from Sigma Chemical Co., St. Louis, MO. 5′-[*p*-(Fluorosulfonyl)benzoyl]adenosine (hydrochloride) (FSA) was also from Sigma Chemical Co. All of the commercial nucleotides were found to be at least 95% pure by thin layer chromatography. 3α,20β-Hydroxysteroid dehydrogenase from *Streptomyces hydrogenans*, with specific activity 12–18 U/mg, was obtained from Boehringer Mannheim Corp.

Methods

Enzyme assays (for 20β activity) were conducted in matched 1 × 1 cm cuvettes at 25 °C with the following solutions added to a final volume of 3.0 mL: 2.6 mL of 0.05 M potassium phosphate buffer, pH 6.5, 0.100 mL aliquot of enzyme solution (225 μg of 3α,20β-HSD in 5.0 mL of 0.05 M potassium phosphate buffer, pH 7.0), 0.200 mL of progesterone (0.1 μmol) in ethanol, and 0.100 mL of NADH (0.35 μmol) in 0.05 M potassium phosphate buffer, pH 7.0. The slope of the initial linear decrease in absorbance at 340 nm (due to oxidation of NADH) as a function of time was used to calculate enzyme activity. Assays were performed in triplicate at 25 ± 1°C in a Beckman Model 25 recording spectrophotometer. Assays for 3α enzyme activity were performed on 0.300-mL aliquots from the enzyme incubation solutions according to the above procedure except that 0.200 mL of 5α-dihydrotestosterone (0.5 μmol) in ethanol was used in place of progesterone.

Affinity-Labeling Experiments. FSA (12.25 mg; 25 μmol) dissolved in ethylene glycol monomethyl ether (1.0 mL) served

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[1] Abbreviations used: FSA, 5′-[*p*-(fluorosulfonyl)benzoyl]adenosine; 3α,20β-HSD, 3α,20β-hydroxysteroid dehydrogenase; NADH, nicotinamide adenine dinucleotide (reduced form); NAD, nicotinamide adenine dinucleotide (oxidized form); ADPR, adenosine 5′-diphosphate-ribose; ADP, adenosine 5′-diphosphate; AMP, adenosine 5′-monophosphate; NMN, nicotinamide mononucleotide (oxidized form); NMNH, nicotinamide mononucleotide (reduced form).

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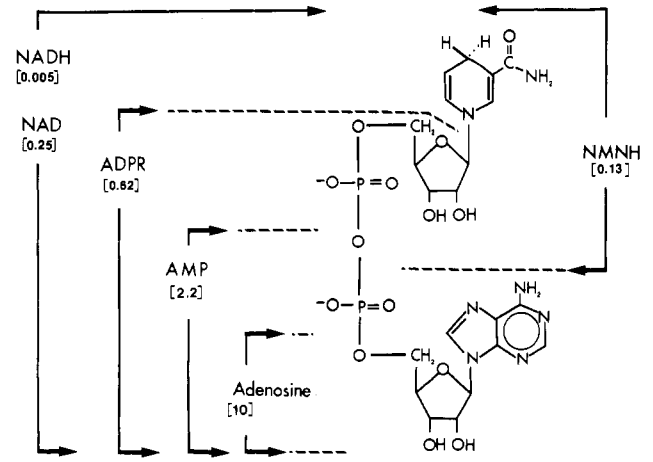

FIGURE 1: Map of nicotinamide adenine dinucleotide components which competitively inhibit NADH binding to 3α,20β-hydroxysteroid dehydrogenase. The numbers in brackets represent the corresponding K_i values (in mM) derived from Dixon plots of kinetic measurements described under Methods and Results, and in Table I. ADP [1.8 mM] and NMN are not represented here. Additional data are provided in Table I.

as the stock solution. In most of the experiments 125 μM solutions of FSA were incubated with 3α,20β-HSD (0.45 μM) in 5.0 mL of 0.05 M phosphate buffer at pH 7.0, 0 °C. A similar solution containing adenosine (equimolar concentration relative to FSA) and 3α,20β-HSD (0.45 μM) served as the control. The time at which FSA was added to a solution containing 3α,20β-HSD was taken as time zero. Aliquots from the incubation mixtures were withdrawn at 15–20-min intervals and assayed for 3α or 20β activity. For each set of experimental conditions, whether simultaneous assays of 3α and 20β activities or assays of the two activities from two consecutive enzyme inactivations were carried out, practically identical results were obtained. The results are summarized in Figure 2 and in Table I.

Results

Competitive Inhibition of 3α,20β-HSD with NADH, NAD, NMNH, ADPR, AMP, ADP, Adenosine, or NMN. Enzyme assays for 3α or 20β activity were conducted under conditions described under Methods. Progesterone (20β activity) was the fixed substrate at 0.1 mM. NADH concentrations were varied between 5 and 20 μM. The inhibitor concentrations were varied from 10 μM to 10 mM. The velocity of the enzyme reaction under these assay conditions was calculated from the slope of the initial linear decrease with time of light absorbance at 340 nm, and assays were run in triplicate. Dixon plots of the data from these experiments showed the inhibition to be competitive. The results are mapped in Figure 1 and summarized in Table I. NMN, at its solubility-limiting concentration of 10 mM, had no influence on the rate of NADH oxidation by 3α,20β-HSD with progesterone under the assay conditions (described above).

Inactivation of 3α,20β-HSD with FSA in the Presence of Progesterone, NADH, NAD, NMNH, ADPR, AMP, ADP, Adenosine, or NMN. Solutions of 3α,20β-HSD (0.45 μM) in 5 mL of 0.05 M phosphate buffer at pH 7.0 and 0 °C were incubated with FSA (125 μM) and varying concentrations of progesterone, NADH, NAD, NMNH, ADPR, AMP, ADP, adenosine, or NMN. Aliquots, periodically withdrawn from the incubation mixtures, were assayed for 3α and 20β activities. First-order kinetics of inactivation wherein 3α and 20β activity decreased simultaneously at practically equal rates under each set of experimental conditions were consistently obtained. The results from these experiments are presented in Figure 2 and Table I.

Table I: Competitive Inhibition vs. Inactivation of 3α,20β-Hydroxysteroid Dehydrogenase by FSA.

inhibitors	K_i[a] (mM)	[I][b] (μM)	k_{calcd}[c] (min^{-1} × 10^3)	k_{obsd}[d] (min^{-1} × 10^3)
NADH	0.005[e]	0		2.30
		2.5	1.5	0.89
		25	0.38	0.24
NMNH		0		2.20
	0.13	250	[0.75][f]	2.19
		1000	[0.25][f]	2.25
(NMNH + AMP)	(0.05)			
NAD	0.25[e]	0		2.40
		2.5	2.30	2.2
		25	2.18	1.48
		250	1.20	0.95
ADPR	0.62	0		2.0
		25	1.99	1.63
		62.5	1.65	0.94
ADP	1.8	0		2.50
		195	2.19	2.07
AMP	2.2	0		2.00
		250	1.79	0.90
		1000	1.37	0.60
(AMP + NMN)	(0.5)			
adenosine	10	0		2.3
		125	2.27	2.2
		1000	2.09	1.8
NMN		0		2.4
		25		2.35
		1000		2.4
progesterone		125		2.2
5α-dihydrotes-tosterone		250		2.3

[a] K_i values were derived from Dixon plots of the data based on inhibition kinetic experiments in which enzyme assays were conducted with 3α,20β-HSD, progesterone as fixed substrate, NADH as variable cofactor, and variable inhibitor concentrations (see Methods). [b] [I] is the concentration of inhibitor during affinity labeling of 3α,20β-HSD with FSA (see Figure 2). [c] k_{calcd} values were calculated from $k_{obsd}[(\ln 2)/t_{1/2}]$ at [I] = 0 and the corresponding values for K_i and [I] (see Appendix). [d] k_{obsd} represents first-order rate constants derived from plots (Figure 1) of log % enzyme activity vs. time in which 3α,20β-HSD (0.45 μM) was incubated with FSA (125 μM) at pH 7.0 and 0 °C. [e] K_i values were taken from the Ph.D. thesis of Betz (1968). [f] The hypothetical k_{calcd} values were calculated from the K_i and [I] values of NMNH, although the nucleotide did not inhibit affinity labeling by FSA.

Calculation of Inactivation Rate Constants for Affinity Labeling by FSA of 3α,20β-HSD in the Presence of a Nucleotide Component. All of the above mono- and dinucleotides which protected 3α,20β-HSD against affinity labeling by FSA were also competitive inhibitors. We derived an equation from classical equilibrium and enzyme kinetics considerations for calculating the influence of a competitive inhibitor on the first-order inactivation rate constant for affinity labeling (Appendix). The equation

$$k_{calcd} = k_{obsd}\left[\frac{1}{[I]/K_i + 1}\right]$$

was used to calculate the inactivation rate constants (k_{calcd}) shown in Table I. The experimentally derived values (k_{obsd} for [I] > 0) agreed with the k_{calcd} values for the adenosine nucleotides which protected 3α,20β-HSD against inactivation by FSA. The k_{obsd} values for NMNH did not agree with the corresponding k_{calcd} values, suggesting that this mononucleotide and FSA bind to two different domains in the NADH cofactor binding region.

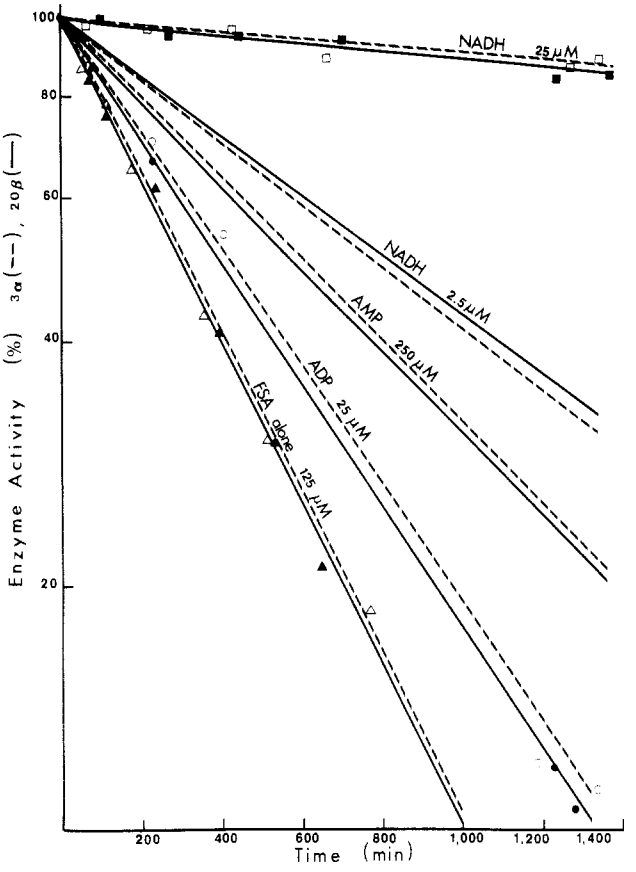

FIGURE 2: Competitive inhibition of 3α,20β-hydroxysteroid dehydrogenase vs. enzyme inactivation by the affinity-labeling nucleoside 5′-[*p*-(fluorosulfonyl)benzoyl]adenosine. The logarithms of the percent 3α (broken line) and 20β (solid line) enzyme activities relative to the control were plotted against the times of incubation. In each experiments, solutions of 3α,20β-HSD (0.45 μM) and FSA (125 μM) in 0.05 M phosphate buffer pH 7.0 at 0 °C were incubated for up to 2400 min, depending on the rate of enzyme inactivation. AMP, ADP, or NADH was added to the incubation mixtures at the concentrations shown. Time zero was taken as the time when FSA was added last to the incubation mixture. The control incubation mixture contained 3α,20β-HSD (0.45 μM) and adenosine (125 μM). Each curve was obtained by least mean squares fit of the averaged data points from at least two experiments. Additional data are provided in Table I.

Discussion

The present investigation was intended to further test our hypothesis that both 3α and 20β activities share a common site of 3α,20β-HSD. Earlier, steroid substrates containing bromoacetoxy side chains affinity-labeled the steroid binding region, causing simultaneous disappearance of the two activities of 3α,20β-HSD (Sweet & Samant, 1980a; Sweet, et al., 1980). We subsequently found that FSA affinity-labeled the NADH cofactor binding region in the active site of 3α,20β-HSD (Sweet & Samant, 1980). In the present investigation, FSA caused both 3α and 20β activities to disappear at equal rates under a wide variety of experimental conditions (Figure 2). These results confirmed that both 3α and 20β activities share the same active site of 3α,20β-HSD.

In the present investigation, NADH and NAD most effectively protected 3α,20β-HSD against inactivation by FSA (Table I). No doubt FSA affinity-labeled a domain within the cofactor binding region at the active site of 3α,20β-HSD. The adenosine-derived components of NADH also protected 3α,20β-HSD against affinity labeling by FSA. The results, summarized in Figure 2, and in Table I, show that the order of this protection was NADH > NAD > ADPR > ADP > AMP > adenosine. These data suggested that in addition to NADH and NAD, adenosine and its 5′-phosphate esters competed with FSA for binding to an adenosine binding domain at the active site of 3α,20β-HSD. Reduction of the first-order rate constant for affinity labeling by FSA was quantitatively dependent on the K_i values and concentrations of the adenosone mononucleotide components (Table I). Thus the dinucleotides and the adenosine mononucleotides AMP, ADP, and ADPR must protect 3α,20β-HSD against FSA by occupying the same domain. Conversely, the presence of FSA at the active site prevented cofactor binding which caused the simultaneous loss of 3α and 20β enzyme activities.

The 5′-phosphate ester group on adenosine (i.e., AMP) *increased* the affinity of the nucleotide for the NADH binding region of 3α,20β-HSD, about 5-fold (Figure 1 and Table I). Similar enhancement of binding to the cofactor sites of adenosine vs. AMP due to the 5′-phosphate group have recently been observed with broad bean alcohol dehydrogenase (Leblova et al., 1979) and also with mitochondrial malate dehydrogenase (Oza & Share, 1973). But the 5′-diphosphate ester group of ADP relative to AMP only slightly increased the affinity of the nucleotide for 3α,20β-HSD. However, presence of the additional ribose group of ADPR caused a 3- to 4-fold enhancement of binding relative to AMP and ADP on the basis of K_i values (Figure 1 and Table I). Similar effects with ADPR have been encountered in a study with NAD-dependent malate dehydrogenase (Oza & Share, 1973). The AMP and ADPR components probably promote binding of NADH by anchoring it to the adenosine domain in the cofactor region at the enzyme active site.

NMN and NMNH did not protect 3α,20β-HSD against affinity labeling by FSA even at a concentration of 10 mM. Evidently, the nicotinamide nucleosides and FSA bind to two different domains in the NADH binding region. Moreover, NMN was not a competitive inhibitor of 3α,20β-HSD, suggesting that in contrast to NMNH the oxidized nicotinamide nucleotide component does not bind to the cofactor binding region. These results with NMN are consistent with those recently reported by Danenberg et al. (1978), who elegantly demonstrated that the positive charge on the pyridinium (i.e., oxidized nicotinamide) moiety of NAD is responsible for *reducing* its binding to liver alcohol dehydogenase by nearly *3 orders of magnitude*.

Cooperation in binding to the active site of 3α,20β-HSD between NMN with AMP, and also NMNH with AMP, is interesting. The apparent K_i value of AMP underwent a 4.4-fold reduction in the presence of NMN, from 2.2 mM to 0.05 mM. Similarly, AMP caused a 2.5-fold reduction in the apparent K_i value of NMNH, from 0.125 mM to 0.05 mM (Table I). Considering K_i values as binding affinities, these results imply that NMN can bind to the enzyme active site *only* in the presence of AMP. Furthermore, AMP enhanced the binding of NMNH to the nicotinamide domain at the active site of 3α,20β-HSD. This seemed to contradict results with glutamate dehydrogenase in which ADP (50 μM) inhibited reduction of NMN (4.5 mM) at a NAD cofactor "subsite" (Cross & Fisher, 1970; Pantaloni & Dessen, 1969). However, 3α,20β-HSD neither oxidized NMNH nor reduced NMN under a wide variety of our conditions. The present conclusions are derived from the mononucleotide's inhibition of NADH oxidation by 3α,20β-HSD. Mixtures of NMN and AMP competitively inhibited 3α,20β-HSD at many times lower concentration than AMP alone, even though NMN by itself was not an inhibitor. Therefore, the present results suggest that NMN with AMP and NMNH with AMP mu-

tually reinforce their binding to the cofactor region at the active site of 3α,20β-HSD.

FSA served as a useful model to study cofactor binding and promotion of 3α,20β-HSD activity. Adenosine must first anchor the 5′-*p*-(fluorosulfonyl)benzoyl reagent group to the cofactor region before it can react with an amino acid residue at the enzyme active site. The AMP or ADPR components of NAD or NADH similarily must anchor the nicotinamide group to the adenosine domain in the cofactor binding region. Then the nicotinamide "reagent group" carries out the hydrogen-transfer reaction in its domain near the steroid binding region at the active site of 3α,20β-HSD. This concept may be useful to describe how NAD and NADH (also NADP and NADPH) bind and promote the catalytic activity of other oxidoreductases.

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Appendix

For the equilibrium reaction between the enzyme and competitive inhibitor

$$E + I \underset{}{\overset{K_i}{\rightleftharpoons}} EI \qquad (1)$$

which competes against the *affinity-labeling* reaction

$$E + L \rightleftarrows EL \xrightarrow{k} E\text{–}L \qquad (2)$$

E = enzyme, I = competitive inhibitor, EI = complex, L = affinity label, EL = complex, and E–L = inactivated enzyme. Treating the affinity-labeling reaction as a classical first-order kinetic enzymic process

$$k = V_{max}/K_m \qquad (3)$$

where k is the inactivation rate constant. From classical competitive inhibition kinetics

$$K_{m,app} = \frac{K_m}{K_i}[I] + K_m \qquad (4)$$

where K_m represents the Michaelis constant in eq 3 and $K_{m,app}$ is the constant corresponding to the effect of a competitive inhibitor on the "enzyme reaction" at a concentration of [I] and with constant K_i. Since V_{max} remains constant for enzyme reactions in the presence of a competitive inhibitor, the altered first-order affinity-labeling rate constant due to the presence of an inhibitor can be derived from eq 3 and 4

$$k_{calcd} = \frac{V_{max}}{K_{m,app}} = \frac{V_{max}}{K_m}\left[\frac{1}{[I]/K_i + 1}\right] = k_{obsd}\left[\frac{1}{[I]/K_i + 1}\right]$$

The k_{calcd} values in Table I were calculated with eq 5 from k_{obsd} (at [I] = 0) and the appropriate K_i and [I] values.

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