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Characterization of Pyridine Nucleotide Binding Site of UDP-glucose 4-Epimerase from *Saccharomyces fragilis**

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UDP-glucose 4-epimerase from *Saccharomyces fragilis* has 1 mol of NAD firmly bound per mol of the dimeric apoenzyme. This prevents a direct study of the coenzyme binding site of the protein. Dissociation of the dimer with *p*-chloromercuribenzoate and its reconstitution with exogenous NAD or one of its analogues and 2-mercaptoethanol provides an indirect method of study of the site.

Depending on the reconstitution properties, the analogues can be classified in the following groups: (i) analogues that have no affinity for the site; (ii) analogues that have affinity but are not incorporated into the apoenzyme; (iii) analogues that produce catalytically inactive holoenzymes; and (iv) analogues that produce catalytically active holoenzymes. Minimum structural requirements that lead to affinity for the coenzyme site and to binding to the apoenzyme can also be discerned from these studies. Reconstitution with etheno-NAD, a fluorescent analogue of NAD, indicates the presence of a hydrophobic pocket for the adenosine subsite.

UDP-glucose 4-epimerase (E C 5.1.3.2; hereafter called epimerase) is the prototype of a number of epimerase (1) and also of some other apparently dissimilar enzymes (2) that obligatorily need nicotinamide adenine dinucleotide as a coenzyme and mediate catalysis through an oxidation-reduction mechanism. The enzyme has been extensively purified from different sources (3–6) but most of the mechanistic studies have been carried out either with the *Saccharomyces fragilis* enzyme or with the *Escherichia coli* enzyme. Both of these enzymes are dimeric holoenzymes with 1 mol of the coenzyme, tightly but noncovalently bound per mol of the dimeric apoenzyme (1, 7). UDP-4-ketohexose and NADH have been clearly identified as enzyme-bound reaction intermediates for the catalytic pathway (1, 7). The mapping of the active site has also been initiated for the yeast enzyme, and two conformationally vicinal sulfhydryl groups have been located in the catalytic region of the active site (8). No report has, however, been published on the nature of the pyridine nucleotide binding site of the yeast or the *E. coli* enzyme. The strong and irreversible binding of the coenzyme to apoenzyme prevents any direct study of this site. The dissociation of the dimeric holoenzyme on titration with *p*-chloromercuribenzoate into monomeric subunits and free coenzyme and its subsequent reconstitution with exogenously added coenzyme (9–11), however, provides an indirect method for the study of the pyridine nucleotide binding site. Employing analogues of NAD as probes in such a reconstituting system, we have now been able to make an initial characterization of the coenzyme binding site of the epimerase from *S. fragilis*.

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EXPERIMENTAL PROCEDURES

Materials—NAD and its various analogues were purchased from P-L Biochemicals. All other biochemicals including NADP, NADH, ADP-ribose and NMN were purchased from Sigma. Nicotinamide [U-^{14}C]adenine dinucleotide (299 mCi/mmol) was purchased from Radiochemical Centre. [^{14}C]NADH was obtained by quantitative reduction of [^{14}C]NAD with alcohol dehydrogenase (12).

UDP-glucose 4-epimerase from *S. fragilis* was purified according to the method of Darrow and Rodstrom (4). The purified enzyme had a specific activity of 14–18 units per mg of protein, where a unit was defined as the amount of enzyme that catalyzed the conversion of 1 μmol of UDP-galactose to UDP-glucose per min at 27 °C.

The enzyme was also purchased from Sigma for some studies. All other enzymes used for this work were obtained from the above source.

Assay Method—UDP-glucose 4-epimerase was assayed in a coupled assay system with UDP-glucose dehydrogenase as the coupling enzyme. The enzyme was also assayed by a two-step assay method where the role of exogenously added NAD had to be completely excluded. Both the assay methods have been described earlier (13). The two-step assay was routinely used to confirm the results obtained with the coupling assay system.

Concentration of *p*-chloromercuribenzoate was calculated from its extinction coefficient of 1.7×10^4 at 232 nm. Protein for epimerase was estimated by measuring absorption at 280 nm following the values for epimerase given by Darrow and Rodstrom (4) or by colorimetric method (14). Molecular weight of yeast epimerase was assumed to be 125,000 (4) for various calculations.

Fluorimetric Methods—Fluorescence was measured with a Perkin-Elmer spectrofluorometer model MPF-44A. The solvent glycylglycine buffer was found to have a minor residual fluorescence. No attempt was made to remove this fluorescence.

RESULTS

Analogues Producing Catalytically Active Holoenzymes—Originally Darrow and Rodstrom (9) and later Bertland and Bertland (10) showed that on quantitative titration with *p*-chloromercuribenzoate the dimeric holoenzyme from *S. fragilis* dissociated into monomers and a molar amount of coenzyme was released from the native holoenzyme. Incubation with 2-mercaptoethanol reassociated the monomers but regeneration of partially active holoenzyme strictly depended on the simultaneous presence of 2-mercaptoethanol and exogenously added NAD. Further studies by these workers and also by us (11) have shown that the reconstituted holoenzyme is similar to the native enzyme in every respect except that unlike the native enzyme the reconstituted holoenzyme does not show any blue fluorescence of the native enzyme (excitation: 360 nm; emission: 435 nm) due to the bound coenzyme. The reconstitution system thus provides a generally valid

model for the study of the coenzyme-apoenzyme interactions, even though the exact nature of the fluorophore in the native enzyme or the reason for its lack of regeneration in the reconstituted system is not known at the moment (11).

A large number of analogues of NAD modified either in the pyridine moiety or in the adenine moiety or in both the positions were tested for catalytic activity in the reconstitution system. In each case exogenously added NAD was replaced individually by these analogues. To eliminate the possibility that the molar amount of NAD that was liberated from the native holoenzyme during treatment with *p*-chloromercuribenzoate could be involved in partial reconstitution of the enzyme; a gel filtration step on a Sephadex G-50 column was included before the reconstitution was carried out.

Results in Table I show that of the various analogues tested, only acetylpyridine NAD,[1] thio-NAD, and etheno-NAD were found to produce catalytic holoenzymes. For comparison, the result with aldehyde NAD, an inactive analogue is also included in Table I. As earlier reported by us (11), the NAD-reconstituted enzyme showed about 40% of the activity of the native enzyme even under most optimal conditions. The extent of reconstitution was dependent on the concentration of exogenously added pyridine nucleotide and typical hyperbolic saturation curves were obtained in each of these cases. Approximate dissociation constants, which are numerically equal to half-saturating concentration for maximum catalytic activity are given in Table I. Final relative activities were determined at saturating concentrations of reconstitution for each individual analogue and with the saturating concentration of UDP-Gal as substrate. The K_m for UDP-Gal as substrate was determined in a coupled assay system.

All these analogues were found to be bound to the reconstituted enzyme. This was demonstrated by passing each of these reconstituted enzymes through a second Sephadex G-50 column and then carrying out two-step assays. Under these conditions all the reconstituted enzymes retained their catalytic activities.

Analogues with Affinity for the Pyridine Nucleotide Binding Site—Aldehyde NAD, hypoxanthine NAD, acetylpyridine hypoxanthine NAD, NADP, α-NAD, and NMN failed to show any catalytic activity in the reconstitution system. Lack of catalytic activity, however, did not exclude the possibility that some of these analogues might have significant affinity for the nascent binding site of NAD of the reconstituted apoenzyme. In fact, these analogues might get incorporated into the protein to produce inactive holoenzymes. The possible affinity for the binding site was studied by competition experiments. When NAD and one of its analogues (1:15) were simultaneously present in the reconstitution medium, significantly less reconstitution of NAD, as measured by ultimate catalytic activity, was noted in the presence of most of the analogues. In contrast, some analogues like α-NAD or NMN had no detectable effect on the reconstitution process (Table II). The competitive nature of interaction for the pyridine nucleotide binding site was further demonstrated by varying the concentration of NAD at a fixed concentration of the analogue. The results for such experiments with aldehyde NAD and hypoxanthine NAD as the analogues are shown in Fig. 1.

[1] The abbreviations used are: Acetylpyridine NAD, 3-acetylpyridine adenine dinucleotide; aldehyde NAD, 3-pyridine aldehyde adenine dinucleotide; thio-NAD, thionicotinamide adenine dinucleotide; hypoxanthine NAD, nicotinamide hypoxanthine dinucleotide; acetyl hypoxanthine NAD, 3-acetylpyridine hypoxanthine dinucleotide; NMN, nicotinamide mononucleotide; etheno-NAD, nicotinamide, 1,N^6-ethenoadenine dinucleotide; ANS, 8-anilino-1-naphthalene sulfonate; Cibacron blue, Cibacron blue F3GA.

TABLE I

Properties of the catalytically active native and reconstituted holoenzymes

For reconstitution with NAD, 0.5 unit of epimerase was taken in 0.25 ml of 50 mM glycylglycine buffer, pH 7.7. The enzyme was inactivated completely by addition of 50 nmol of *p*-chloromercuribenzoate. The inactivated enzyme was passed through a Sephadex G-50 column (0.8 × 20 cm) that was equilibrated with 50 mM glycylglycine buffer, pH 7.7, and eluted with the same buffer. 120-μl aliquots of the 1-ml protein-containing fraction were now transferred to several tubes each containing in a total volume of 150 μl, 15 μmol of glycylglycine buffer, pH 7.7, 0.4 μmol of 2-mercaptoethanol, and varying concentrations of NAD. A control without NAD was simultaneously run. Another control of native enzyme that was not originally inactivated by *p*-chloromercuribenzoate was processed through these steps. After incubation at 27 °C for 30 min, 10-μl aliquots from each tube were taken out for the two-step assay systems. The maximum reconstituted activity and half-saturating concentration for maximum activity for NAD were obtained from this experiment. The values for the other analogues were obtained in separate experiments where NAD was replaced by individual analogues. Aliquots for the two-step assay varied according to the activity of the reconstituted enzymes.

Enzyme	Analogue	Maximum activity	Half-saturating concentration for maximum activity	K_m for UDP-Gal
		%	μM	
1. Native	NAD	100		108
2. Reconstituted	NAD	39	8	138
3. -Do-	Acetylpyridine NAD	3.4	250	126
4. -Do-	Thio-NAD	4.2	360	132
5. -Do-	Etheno-NAD	9.6	381	139
6. -Do-	Aldehyde NAD	<0.5		

TABLE II

Competition for the NAD binding site by the analogues

For this experiment 2.1 units of the enzyme in 500 μl of 0.1 M glycylglycine buffer, pH 7.6, were inactivated with 5 μl of 20 mM *p*-chloromercuribenzoate. 25-μl aliquots of the inactivated enzyme were now transferred to several tubes, each containing in a total volume of 100 μl, 2 mM mercaptoethanol, 50 μM NAD, and 0.75 mM of an analogue of NAD as indicated in the table. The tube without any analogue served as the control. After an incubation period of 30 min at 27 °C, 10-μl aliquots were taken for the coupled assay. Wherever necessary two-step assays were also performed. Formation of inactive holoenzymes was tested by methods described in the text. Summarized results are given here for comparison.

Additions	Rate	Formation of inactive holoenzyme
	$(\Delta A/min) \times 10^3$	
1. Control	26.1	
2. Hypoxanthine NAD	15.6	+
3. Aldehyde NAD	3.1	+
4. Acetylpyridine hypoxanthine NAD	20.8	+
5. NADP	16.2	−
6. NADH	13.7	−
7. α-NAD	24.9	−
8. NMN	25.2	−
9. ADP-ribose	1.2	+
10. ADP	25.9	−
11. Cibacron blue	1.0	+

Analogues Forming Catalytically Inactive Holoenzymes—Some of the above analogues which were involved in competition with NAD could also be irreversibly incorporated into the reconstituted dimer. In the absence of radioactive analogues, a direct demonstration of binding was difficult. We

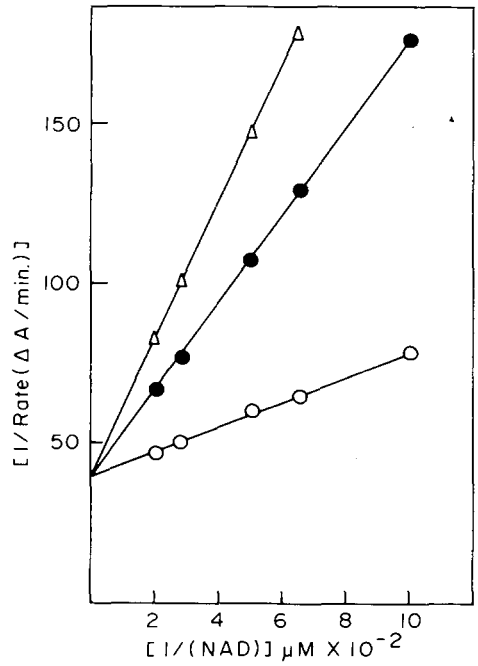

FIG. 1. **Competition of analogues with NAD for the pyridine nucleotide binding site.** For this experiment 1 unit of epimerase was taken in 1.8 ml of 0.05 M glycylglycine buffer, pH 7.7. The enzyme was inactivated by addition of 100 nmol of *p*-chloromercuribenzoate. 100-μl aliquots of the inactivated enzyme were transferred to several tubes each containing in a total volume of 150 μl, 10 μmol of glycylglycine buffer, 0.3 μmol of 2-mercaptoethanol, varying concentrations of NAD, and 50 nmol of hypoxanthine NAD or 20 nmol of aldehyde NAD, as indicated in the figure. After incubation at 27 °C for 30 min, 20-μl aliquots from each tube were taken for assay by the coupled assay system. ○——○, ●——●, and △——△ indicate the presence of only NAD, NAD with hypoxanthine NAD, and NAD with aldehyde NAD, respectively.

could, however, show that once the holoenzyme was formed with an analogue, the analogue could not be displaced by subsequent addition of NAD. Thus, when *p*-chloromercuribenzoate-inactivated enzyme was preincubated with aldehyde NAD and 2-mercaptoethanol, subsequent addition of [^{14}C]NAD failed to show any incorporation of radioactivity in the protein eluant coming in the void volume of a Sephadex G-50 column (Fig. 2). In this case, in the control sample (Fig. 2*A*) on reconstitution with [^{14}C]NAD alone, 0.89 mol of NAD (8050 cpm in protein eluant) was found per mol of the apoenzyme, whereas the experimental sample (Fig. 2*B*) on preincubation with aldehyde NAD and subsequent addition of [^{14}C]NAD showed less than 0.03 mol of NAD (250 cpm in protein eluant) binding per mol of the apoenzyme. Similar experiments were carried out with all the other analogues that had shown affinity for the NAD-binding site. Except for NADH and NADP, these analogues almost completely prevented the incorporation of [^{14}C]NAD into the protein moiety. Nature of interaction of NADH to the reconstituted apoenzyme was assessed in a similar experiment. In this case, in comparison to 0.86 mol of NAD that had bound to the protein molecule in the control, 0.72 mol of NAD was found to be bound to the protein in the experimental sample (data not shown). The slightly less binding of NAD in the experimental sample was possibly due to the competition between NADH and NAD for the coenzyme binding site in the final reconstitution process. An essentially similar result was obtained when NADP was used as the reconstituting analogue. Lack of binding of NADH to the reconstituted protein was also directly demonstrated by using [^{14}C]NADH in the reconstitution medium. No significant protein-bound count was obtained after passing the reconstitution medium through the Sephadex column (data not shown).

The very strong and effectively irreversible binding of the above analogues to produce catalytically inactive holoenzymes

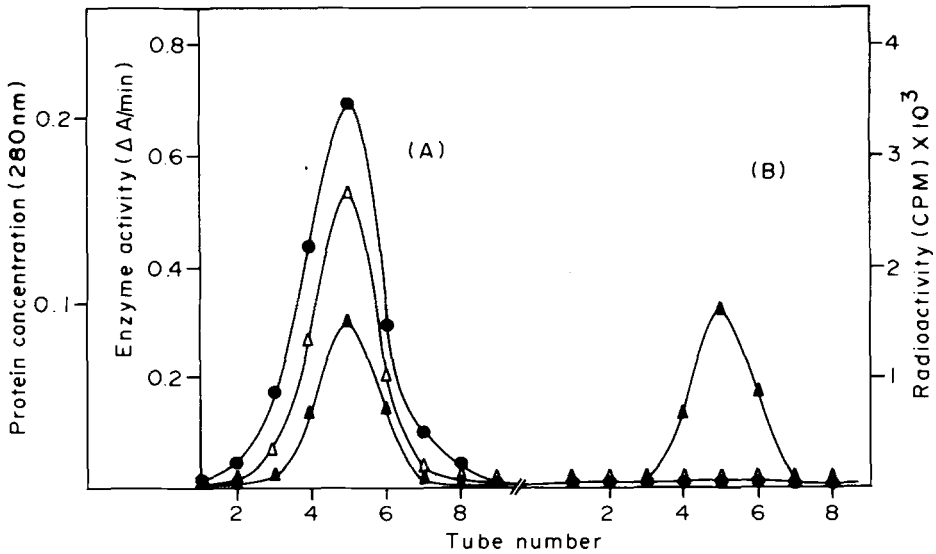

FIG. 2. **Binding of aldehyde NAD with the reconstituted apoenzyme.** 3.3 units of enzyme (0.2 mg of protein) were taken in 200 μl of 0.05 M glycylglycine buffer, pH 7.7. The enzyme was inactivated by addition of 100 nmol of *p*-chloromercuribenzoate. The inactive enzyme was incubated for 30 min at 27 °C by addition of the final concentration of 4 nM of 2-mercaptoethanol and 0.04 mM [^{14}C]NAD containing 2.24 × 10^5 cpm. Then the incubation mixture was passed through a Sephadex G-50 column (0.8 × 20 cm) and eluted with the same buffer. Fractions of 1 ml were collected and in each tube enzyme activity, protein concentration, and protein-bound counts were estimated. Against this control reconstituted system, a parallel experiment was run. In this case, the same amount of inactive enzyme was preincubated with 0.4 mM aldehyde NAD and 4 mM 2-mercaptoethanol for 30 min at 27 °C, and then [^{14}C]NAD of the same quantity as used before was added and incubated for another 30 min. Then the whole mixture was eluted in a Sephadex column (0.8 × 20 cm) as before and the same parameters were measured. *A*, shows the elution profile of the control experiment in the absence of aldehyde NAD. *B*, shows the elution profile of the protein in presence of aldehyde NAD. ●——●, △——△, and ▲——▲ represent protein-bound radioactive count, enzyme activity, and absorbance, respectively.

was further confirmed in the following manner. Each of these analogues (1 mM) was first incubated with the *p*-chloromercuribenzoate-inactivated enzyme and mercaptoethanol (5 mM) for 30 min. The reconstituted protein fraction was then separated from the excess analogue and mercaptoethanol by gel filtration. Addition of fresh NAD (0.1 mM) and mercaptoethanol (5 mM) to an aliquot of the protein fraction failed to reactivate the enzyme indicating the formation of an inactive holoenzyme that had its coenzyme-binding site occupied by the analogue. Control *p*-chloromercuribenzoate-inactivated enzyme sample was first incubated in the presence of mercaptoethanol but without NAD or any of the analogues. Subsequent processing, as in the case of the experimental samples, resulted in considerable regeneration of activity (25%) indicating that in this case the coenzyme binding site was unoccupied and ready to accept NAD.

Environment of Pyridine Nucleotide Binding Site—Fluorescent probes have been widely utilized to monitor hydrophobic regions in proteins and membrane structures. Etheno-NAD is a moderately fluorescent analogue of NAD (15) and has been used by other workers for the study of the active site of many dehydrogenases (16, 17). Since an active holoenzyme could be reconstituted with etheno-NAD (Table I), this provided a direct method to monitor hydrophobicity at the NAD binding site of the enzyme. When the etheno-NAD-reconstituted enzyme was excited at 305 nm, a new fluorescence spectrum with an emission maximum at 396 nm was observed. A NAD-reconstituted control enzyme sample failed to show any fluorescence in this region. Compared to an equimolar amount of free etheno-NAD, there was a blue shift of about 14 nm in the emission maximum and an enhancement of fluorescence by a factor of approximately two was observed (Fig. 3). The fluorescence enhancement was calculated on the assumption that stoichiometry of etheno-NAD binding to the apoenzyme was essentially the same as that for NAD binding. The peak at 342 nm was probably due to the tryptophan fluorescence of the protein itself.

The blue shift and enhancement of fluorescence which are comparable to results obtained with some of the dehydrogenases (16, 17) suggested a hydrophobic milieu for the adenine moiety of the pyridine nucleotide. To check whether this region presents generally nonspecific hydrophobic interaction site, 8-anilino napthol sulfonic acid was used as a probe in the reconstitution medium. Addition of ANS to the reconstituted apoenzyme resulted in an immediate and dramatic enhancement of fluorescence. An enhancement of about 70-fold and a blue shift of about 40 nm were observed. On closer scrutiny, this interaction was found to be unrelated to the pyridine binding site. When the incubation mixture was passed through a long Sephadex G-50 column, the dye was found to be completely separated from the apoenzyme. Moreover, absolutely no competition between the dye and NAD could be demonstrated during the reconstitution process. Finally, just like the reconstituted apoenzyme, both the native holoenzyme and the NAD-reconstituted enzyme showed this characteristic interaction with the dye. Obviously, whatever be the nature of the interaction of ANS with the protein, it is not related to the coenzyme binding site of the molecule. Similar interaction of ANS with epimerase was first noted by Wong and Frey for the *E. coli* enzyme (18). We have also recently made a detailed study of this phenomenon (19).

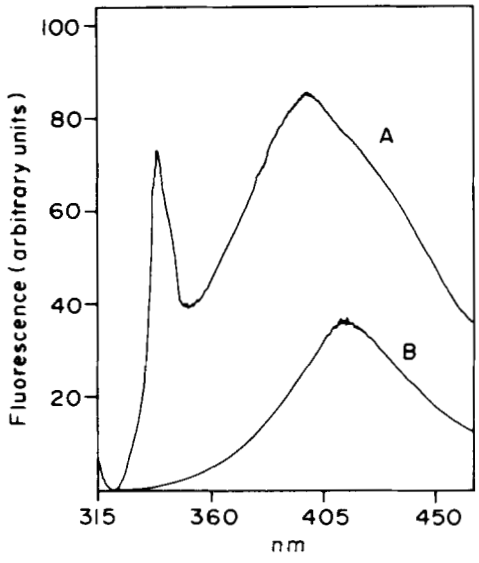

FIG. 3. **Fluorescence spectrum of etheno-NAD-reconstituted enzyme.** 124 μg of *p*-chloromercuribenzoate-inactivated enzyme in 0.05 M glycylglycine buffer was reconstituted with 1 mM etheno-NAD and 4 mM 2-mercaptoethanol. Excess analogue was removed by passing the incubation mixture through a G-50 column. Emission spectrum of an eluant fraction containing 50 μg of protein was recorded in *A*. Spectrum of 0.4 nmol of etheno-NAD in the same volume is presented for comparison (*B*). Excitations were at 305 nm.

DISCUSSION

The reconstituted dimer obtained from the *p*-chloromercuribenzoate-inactivated enzyme provides a model system to study the interaction of the pyridine nucleotide with the native apoenzyme. Certain minimum structural requirements for the interaction can be discerned. α-Linkage between N of pyridine and C_1 of ribose probably presents some steric problems since in contrast to ADP-ribose and β-NAD, α-NAD shows no affinity for the site. Presence of neither of the phosphates alone is sufficient for affinity since neither NMN nor ADP competes with NAD for the coenzyme binding site. In contrast to ADP, ADP-ribose is a strong competitor for NAD and is efficiently incorporated into the apoenzyme to give an inactive holoenzyme (Table II). Obviously, the ribose attached to the pyridine moiety is critically involved in irreversible binding of the coenzyme to the apoenzyme. ADP-ribose was previously shown by Glaser and his group to compete for the NAD-binding site in other oxidoreductases (20) including the liver epimerase where NAD is needed exogenously for catalysis (21).

If α-NAD, NMN, and ADP are the first class of analogues that have no affinity for the coenzyme site, NADH and NADP constitute a second class that shows significant affinity for the site but is not permanently incorporated into the apoenzyme. The lack of binding of NADH is somewhat surprising, since the coenzyme in the native holoenzyme can be reduced easily by sodium borohydride and still found to be retained on the enzyme surface (22). Langer and Glaser (21) had shown that the binding of NADH to the liver apoenzyme is enormously enhanced in presence of oxidized substrate. A similar situation may be operative for the reconstituted enzyme. Alternately, an intermediate stage in binding involving the monomer and the coenzyme may be assumed. NADH or NADP might be competing for the site at this stage, although these analogues are excluded in the final dimeric product. These important questions about the assembly of the holoenzyme need to be studied separately and in considerable detail.

A third class of analogues are those that become tightly bound to the apoenzyme but produce catalytically inactive holoenzymes (Table II and Fig. 1). The minimum requirement for this strong binding is the presence of ADP-ribose moiety

or a similar moiety where adenine is replaced by other purine bases. Cibacron blue was originally regarded as a characteristic probe for the dinucleotide fold in dehydrogenases and a detailed study with this probe has already been reported (23).

The final category of analogues are those that give reconstituted holoenzymes which are catalytically active (Table I). Both thio-NAD and acetylpyridine NAD were earlier shown to be active for several dehydrogenases. However, aldehyde NAD which still retains the carbonyl group attached to the C_3 of the pyridine ring is completely inactive. Similarly hypoxanthine NAD with no modification at the pyridine ring is completely inactive. In contrast, etheno-NAD which also has its modification in the purine moiety is found to be catalytically most active in replacing NAD. Obviously, the requirements for catalysis are stringent, but those cannot be predicted from analogue studies. The binding of the coenzyme to the apoenzyme does not seem to influence the binding of the substrate to the holoenzyme. This is indicated from the fact that even when the enzyme is reconstituted by different catalytically active analogues, the K_m for UDP-galactose remains essentially the same (Table I). Furthermore, ANS which is a substrate site-directed probe for this enzyme (19) gives very similar enhancements and blue shifts both with the free apoenzyme and with the coenzyme-bound reconstituted apoenzyme. Presumably, the incorporation of NAD into the apoenzyme, unlike in many dehydrogenases, does not bring about any drastic conformational change in the substrate binding site of the enzyme.

Fluorescence experiments with etheno-NAD strongly suggest the presence of a hydrophobic milieu for the adenosine subsite of the pyridine nucleotide binding site. Similar hydrophobic pockets have been thoroughly documented for several dehydrogenases by direct x-ray crystallographic and other studies (24). However, since ANS failed to compete with NAD for the site, the specificity in this case may not be as broad as in the case of liver alcohol dehydrogenase or lactic dehydrogenase where such fluorescent aromatic dyes were shown to compete for the adenosine subsite of the coenzyme binding site (25, 26).

Existence of elements of a dinucleotide fold as revealed by Cibacron blue studies (23) as well as the presence of a hydrophobic adenine pocket for the pyridine nucleotide binding site suggests some structural homology in the pyridine nucleotide binding sites between dehydrogenases and this prototype oxidoreductase. This may have interesting evolutionary implications that may only be explored by detailed x-ray studies.

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