INSOLUBILIZED COENZYMES: THE COVALENT COUPLING OF ENZYMATICALLY ACTIVE NAD TO GLASS SURFACES*

Michael K. Weibel**, Howard H. Weetall, and Harold J. Bright***

Department of Biochemistry, School of Medicine
University of Pennsylvania, Philadelphia, Pennsylvania 19104

and:

Research and Development Laboratory
Corning Glass Works, Corning, N. Y. 14830

Received May 21, 1971

NAD was attached to the surface of glass beads by a diazo coupling procedure. The insolubilized NAD was shown to function as a coenzyme in the yeast alcohol dehydrogenase reaction.

Our interest in the immobilization of catalytically active pyridine nucleotide coenzymes stems from two immediate applications of such coenzymes. Firstly, the binary complexes of many pyridine nucleotide linked dehydrogenases have dissociation constants in the range 0.1 to 10 μM. Consequently enzyme purification by nucleotide column affinity may be economically feasible. Secondly, reaction pathways which might be constructed, for example, by microencapsulation of the appropriate enzymes, would probably require the participation of nucleotide coenzymes at one or more steps in the pathway. The conservation and regeneration of the nucleotides within the microcapsule would obviously be of advantage. This might be achieved by covalent attachment of the nucleotide coenzyme to a soluble, but non-diffusable, macromolecule in a manner that would allow the nucleotide to retain adequate binding and catalytic properties.

To our knowledge no documented experimental evidence for a catalytically active, insolubilized coenzyme has been reported. We shall present here a method for modifying NAD by covalent coupling to glass and show that the modified nucleotide exhibits redox activity in the reaction catalyzed by YADH.

---

*This project was supported by Grant GK-27693X, National Science Foundation and by Grant GM-11040, National Institutes of Health, U.S.P.H.S.

**To whom inquiries should be addressed. Postdoctoral Trainee of the U.S.P.H.S., Training Grant AM-5150.

***Research Career Development Awardee, K3-GM-34960, U.S.P.H.S.

Abbreviations used are: YADH, yeast alcohol dehydrogenase; BSA, bovine serum albumin; NAD, nicotinamide adenine dinucleotide.

Materials and Methods: YADH (EC 1.1.1.1.) was obtained from Boehringer Mannheim Corp. and used without further purification. All solvents, reagents and substrates were either C.P. or analyzed grade. NAD and NADH (Grade III) were obtained from Sigma Chemical Co. The δ-aminopropyl triethoxy silane (A-1100) was used as obtained from Union Carbide.

Porous glass particles (40/60 mesh), with an average pore diameter of 550 Å, were obtained from Corning Glass Works. Solid glass beads (0.25-0.33 mm) were obtained from Bronwill Scientific Co. These beads were used as an inorganic support for the coupling procedure. The surface of the glass beads was cleaned and etched by brief exposure to a concentrated HF acid solution and by further incubation in hot 10 N NaOH for 1 hr.

Coupling of NAD to the glass surface was carried out according to the diazotization procedure of Weetall (1). Approximately 200 g of the prepared glass beads were mixed with 250 ml of dry toluene containing 10% (by weight) A-1100 silane and the slurry refluxed for 24 hr. The glass beads were washed with toluene and acetone, then dried at 60° C in a vacuum oven. The δ-amino group was acylated by refluxing a slurry of the silanized glass beads for 24 hr. in 250 ml chloroform containing 10% (by weight) p-nitrobenzoyl chloride and 10% (by weight) triethyl amine. The resulting acylated glass beads were washed thoroughly with chloroform and dried at 60° C in a vacuum oven. The aryl nitro group was reduced to an aryl amine by refluxing a slurry of the glass beads for 1 hr. in 250 ml aqueous solution of 10% (by weight) sodium dithionite. The glass beads now containing the coupling reagent were washed with water and dried. At this stage the beads are conveniently stored as the subsequent diazotization and coupling procedure are done rapidly to avoid deterioration of the diazonium salt intermediate.

Typically 50 g of the glass beads containing the coupling reagent were diazotized by reaction with 100 ml of 2 N HCl containing 2.5 g sodium nitrite. The reaction was carried out at 0° C in a filter flask which was continually aspirated to remove evolved NO_2. After 20 minutes the reaction slurry was filtered. The diazotized glass beads were washed with 500 ml of cold, aqueous 1% (by weight) sulfamic acid to remove traces of sodium nitrite. The glass beads were then immediately slurried in 50 ml of 10^{-2} M NAD in 0.1 M Tris-HCl, pH 8.5. The coupling reaction was carried out at 25° C. The reaction was monitored by withdrawing aliquots of the supernatant solution and measuring the decrease in absorbance of the diluted sample at 260mμ. Usually the reaction was completed in 20 minutes. Evidence of significant coupling to NAD is indicated by the appearance of a pronounced yellow tinge to the beads. The beads at this stage contain both physically adsorbed and covalently coupled NAD. The adsorbed NAD can be completely removed by thoroughly

washing with 0.01 M phosphate buffer, pH 8.5. In the case of the porous glass particles small but measurable, amounts of NAD were slowly released even after thorough washing. This released NAD could interfere with the sensitive assay for enzymatic activity. For this reason solid glass beads were used in experiments to detect active, coupled NAD in spite of the considerably reduced surface area per ml of bed volume. In practice, however, the porous glass is superior as a support due to its very large surface area available for coupling (100 meters^2/g). The same coupling procedure is used for porous glass particles except the initial weight of the glass is 40 g.

The assay for active, covalently coupled NAD is based upon the equilibrium reaction catalyzed by YADH in equation (1) and involves poising the equilibrium in such a manner that it is sensitive to moderate pH changes.

$$CH_3CH_2OH + NAD^+ \underset{(ADH)}{\overset{K_H}{\rightleftharpoons}} CH_3CHO + NADH + H^+ \qquad (1)$$

The value of K_H is 1.15×10^{-11} M (2). For experimental conditions where $[CH_3CH_2OH] = 0.10$ M >> [NAD], [NADH] and $[CH_3CHO]$, the equilibrium expression is

$$K_H[CH_3CH_2OH] = [CH_3CHO][NADH][H^+]/[NAD] = 1.15 \times 10^{-12} \qquad (2)$$

It can be calculated from equation (2) that essentially all the coenzyme would remain reduced in a system at pH 8.5 containing catalytic quantities of YADH, 0.10 M ethanol and 10^{-5} M NADH if the concentration of acetaldehyde is less than 10^{-4} M. However, upon shifting the pH to 6.5 the equilibrium responds by quantitatively converting either acetaldehyde or NADH (depending upon which is limiting) to ethanol or NAD, respectively. In addition to using the pH dependent equilibrium of equation (1) as a sensitive assay for acetaldehyde, it may be used in conjunction with the immobilized NAD to generate acetaldehyde at pH 8.5.

Typically the generation and detection of acetaldehyde was carried out as follows. Three ml of pH 8.5, 0.01 M phosphate buffer containing 0.10 M ethanol, was added to a 5 ml wet bed volume of the NAD beads and incubated for 1 hr. A 1 ml aliquot was removed and examined spectrophotometrically at 260mμ to ensure that no free NAD had been released, the limit of detection being 10^{-7} M. Appropriate control incubations with enzyme and BSA, but without ethanol, did not produce any spectrophotometrically detectable release of NAD. Therefore our results cannot be attributed to the release or production of soluble NAD. The beads were washed and a fresh 3 ml addition of the buffered 0.10 M ethanol solution was added. Thirty μl of a 1:250 dilution of the Boehringer YADH suspension in 0.1 % BSA was added and the vial capped. After 1 hr a 1 ml aliquot was transfered to a semimicro cuvette. Ten μl of 10^{-3} M NADH was added and the absorbance at 340mμ recorded. A pH jump from 8.5 to

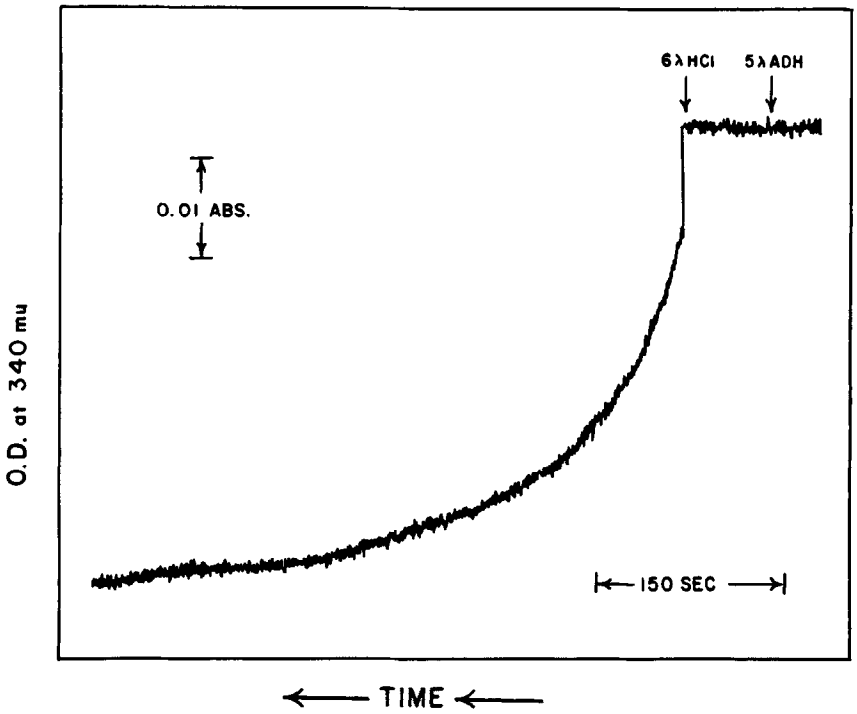

Figure 1. Spectrophotometric assay of acetaldehyde generated by reaction of insolubilized NAD with 0.10 M ethanol in 0.01 M potassium phosphate, pH 8.5 in the presence of YADH. The initial baseline results from the addition of 10 µl of 10^{-3} M NADH to 1 ml of the reaction supernatant solution as described in the text. After the addition of YADH as indicated, no reaction is observed until the pH is jumped from 8.5 to 6.5 by the addition of a calibrated amount of HCl. The decrease in absorbance indicates 7×10^{-6} M acetaldehyde present. Temperature 25° C. The time course of this reaction plots as a first order process.

6.5 was rapidly achieved by the addition of 6 µl of calibrated dilute HCl and 5 µl of the diluted YADH added. Figure 1 shows the results of this experiment. The absorbance change indicates at least 7×10^{-6} M acetaldehyde present. Considering the bead bed was only about 60 % of the total volume in the vial, the effective concentration of NAD in the bed was at least 10^{-5} M. The addition of enzyme to the cuvette requires comment as YADH should be present in the reaction slurry supernatant solution. We have found that additions of very dilute enzyme are removed by adsorption or inactivation at pH 8.5. However, this inactivation or adsorption is slow enough that appreciable reaction takes place before all active enzyme is removed from the supernatant solution.

Upon refining our techniques for coupling NAD we have been able to generate considerable quantities of acetaldehyde. The YADH reaction was performed in 0.1 M phosphate and 15 µl of the undiluted Boehringer YADH suspen-

sion added directly to the reaction mixture. In this case the amount of acetaldehyde generated was sufficiently large that the equilibrium at pH 8.5 no longer prevented the conversion of added NADH to NAD. For this reason the pH jump from 8.5 to 6.5 was initiated before the addition of NADH. The absorbance decay was monitored after each of five additions of 0.12 μmole NADH to 1 ml of the supernatant solution at pH 6.5. The sum of the absorbance changes observed was 3.7, indicating 6×10^{-4} M acetaldehyde detected. This concentration of acetaldehyde would correspond to a minimum value of about 10^{-3} M for the effective concentration of active, coupled NAD in the bead bed. The titration was not carried out to completion because the large quantity of NAD generated would not allow a quantitative end point to be achieved.

We have observed that it is possible to recycle the beads in NADH form back to NAD by using 0.10 M acetaldehyde at pH 6.5. Unfortunately an assay for generated ethanol similar to that for acetaldehyde is not easily achieved due to the equilibrium perturbation by the high concentration of acetaldehyde. However, NAD beads which have been exhaustively converted to the NADH form are able to generate large quantities of acetaldehyde after the recycling reaction.

Discussion: It has not yet been possible to establish the position on the NAD molecule at which coupling occurs. However, the positively charged pyridine ring is an unlikely candidate because the coupling involves electrophilic attack by the diazonium compound. On the other hand, certain purine derivatives undergo diazo coupling (3). In particular, diazonium salts couple to 2,6dihydroxypurine at position C(8), whereas both 6,8 and 2,8-dihydroxypurine are unreactive. Furthermore, molecular orbital calculations (4) of electron density at purine carbon atoms show the highest electron density to be at C(8), the order being C(8) > C(6) > C(2). These facts favor C(8) as the most probable site for diazo coupling to the adenine nucleus of NAD.

Attempts of this kind to immobilize coenzymes and other important ligands for macromolecules will become more rational as more is known about the structures of the complexes involved. Our own strategy has been influenced by the structure of the binary NAD complex of lactate dehydrogenase as revealed by high resolution x-ray crystallography (5). In this complex, the nicotinamide moiety is buried within the protein molecule, whereas portions of the adenine nucleus, including the C(8) atom, appear to be reasonably accessible to solvent. If this is also true in the binary complex of YADH, and if, as we believe, diazo coupling has occured at the C(8) of the adenine nucleus, then it might be expected that the stereochemical conditions for the redox process would not be seriously affected. We are presently attempt-

ing to determine the effects of the coupling of NAD upon the kinetic and binding parameters of the system and to establish whether insolubilized NAD is functional in other dehydrogenase reactions.

References

1. Weetall, H.H., Science, 166, 615 (1969).
2. Racker, E., J. Biol. Chem., 184, 313 (1950).
3. Acheson, R. M., Heterocyclic Compounds, 2nd edition, John Wiley Interscience, N.Y. (1967), p365.
4. Lykos, P. G. and Miller, R. L., Tetrahedron Letters, 23, 1743 (1963).
5. Adams, M. J., McPherson, A., Rossmann, M. G., Schevitz, R. W. and Wonacott, A. J., J. Mol. Biol., 51, 31 (1970).