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Synthesis of fluorinated NAD as a soluble coenzyme for enzymatic chemistry in fluorous solvents and carbon dioxide

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Abstract—The synthesis of the coenzyme nicatinamide adenine dinucleotide (NAD) with an covalently attached fluorinated polymer is reported. The fluorinated NAD (FNAD) was rendered soluble in both fluorous solvents and liquid carbon dioxide due to the attachment of a perfluoropolyether. The enzyme horse liver alcohol dehydrogenase (HLADH) was active in catalyzing oxidation/reduction reactions using FNAD as a soluble coenzyme in a fluorous solvent, methoxynonafluorobutane (HFE), and liquid carbon dioxide. In both solvents, the activity of HLADH using FNAD was greater than the same molar amount of unmodified (insoluble) NAD, indicating that a soluble coenzyme results in more efficient reactions.

1. Introduction

Fluorous solvents, known as perfluorinated solvents, perfluorocarbons (PFC), or just fluorocarbons, are characterized by high density, high stability, and low solubilities in both water and most organic solvents.[1,2] Fluorous solvents as reaction media are of growing interest in that they are considered environmentally benign due to their unique properties. For example, fluorous solvents exhibit low toxicity and are used in many biomedical and clinical applications.[3] They are easy to separate from both aqueous and organic solvents due to their immiscibility in both;[4] however, many organic/fluorous mixtures may become miscible at increased temperatures.[5] Finally, they do not deplete the ozone layer;[6] although one drawback is that they are potent greenhouse gases.[7]

Synthesis in fluorous biphasic mixtures (FBS) is an emerging field that takes advantage of the benefits of fluorous solvents. This process involves catalysis in two immiscible phases, a fluorous and a non-fluorous phase. The catalyst is totally soluble in the fluorous phase while the substrates and products prefer the non-fluorous phase. After reaction, the product is recovered in the non-fluorous phase and the catalyst in the fluorous phase, which then can be recycled. FBS allow easy product separation and recycle of catalysts. Other promising technologies using fluorous solvents include fluorous separations, fluorous synthesis, and fluorous mixture synthesis.[8] In these applications, the catalyst, reagent, or substrate is rendered soluble in a fluorous phase by attaching a fluorinated 'ponytail' to the compound.

It would be interesting to combine the 'green' benefits of fluorous solvents with that of green catalysts (enzymes) to expand non-aqueous biocatalysis into environmentally benign solvents. Enzymes can be considered environ-

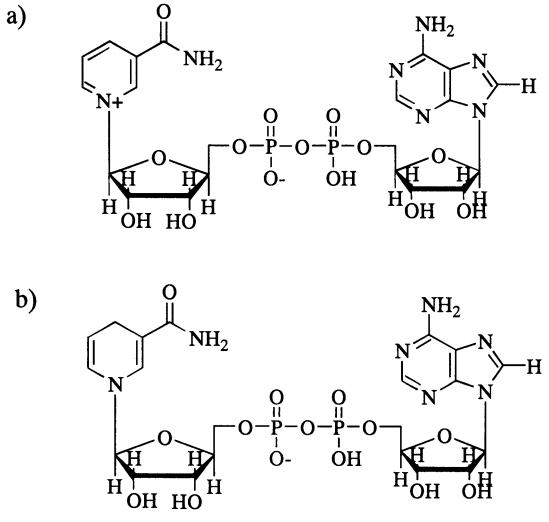

Figure 1. (a) Structure of NAD, (b) structure of NADH.

Keywords: nicatinamide adenine dinucleotide; reduced nicatinamide adenine dinucleotide; alcohol dehydrogenase; horse liver alcohol dehydrogenase; oxidation/reduction; fluorous solvents; carbon dioxide; solubility; high pressure.

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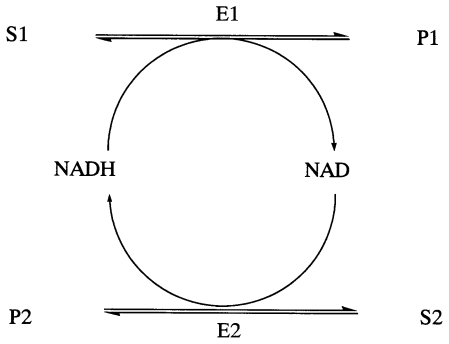

Figure 2. In situ enzymatic recycling of NAD/NADH.

mentally benign catalysts in that they are derived from biological systems, have very high activity, and are selective in the reactions that they catalyze.

Oxidoreductases, commonly known as dehydrogenases, catalyze oxidations and reductions. Dehydrogenases require the participation of nicatinamide adenine dinucleotide (NAD) in the catalysis of these reactions. NAD (Fig. 1(a)) accepts a hydride ion from the substrate, being reduced to NADH (Fig. 1(b)) with the concurrent oxidation of the substrate to product:

$$AH_2 + NAD \rightleftharpoons A + NADH + H^+$$

where AH_2 is the reduced substrate and A is the oxidized product. NAD is required in stoichiometric amounts, but it can be regenerated after it participates in a reaction. For example, in situ enzymatic recycling of NAD is common. Once NAD has been reduced to NADH, it can be regenerated back to NAD by providing a second reaction (Fig. 2). One enzyme (E1) oxidizes NADH to NAD while a second enzyme (E2) reduces NAD back to its original state. At the same time, substrate (S1) is reduced to a product (P1), while a second substrate (S2) is oxidized to a second product (P2). Two separate enzymes can be employed to catalyze the reactions, but sometimes only one enzyme is necessary, as long as the enzyme has broad substrate specificity.

When NAD is used with an enzyme in an organic solvent, both NAD and the enzyme are insoluble. The enzyme and NAD are therefore typically lyophilized together; nevertheless, they are surrounded by what is termed 'essential water', or the bound water that is required for the enzyme to function in the organic solvent.[9] Once NAD participates in a reaction, it is reduced to NADH (or vice versa). It has been hypothesized that NAD(H) must be released from the active site in order for it to be regenerated,[10] but since NAD(H) is not soluble in organic solvents, NAD(H) will not likely leave the active site as required. If NAD(H) is hindered from leaving the active site, the enzyme molecule will become inactive, given that the active site is not free to bind another NAD(H) molecule to continue the reaction. This has been shown to be true for a two-enzyme regeneration reaction.[11] NAD(H) would not traverse the organic phase to participate in both reactions (Fig. 2) if two enzymes (E1 and E2) were lyophilized separately; the insolubility of the coenzyme in the organic solvent prevented the regeneration of the coenzyme. However, Deetz et al. showed that both reactions occurred when the two enzymes and NAD were lyophilized together.[11] They suggested that the essential water bound to the lyophilized solid containing both enzymes offered a layer of hydration so that the cofactor could be released from the active site of E1 to migrate to the active site of E2 through the hydration layer. When the enzymes were lyophilized separately, the two enzymes did not share a hydration layer; therefore, NAD(H) could not travel from E1 to E2 through the organic solvent. We propose to overcome the limitation of NAD(H) transport in organic solvent by derivatizing NAD(H) to affect solubility. Even if a one-enzyme recycling system is used (or if two enzymes are lyophilized together), solubilization of the coenzyme should increase reaction efficiency.

Covalent immobilization of NAD onto polymers has been accomplished previously, specifically to facilitate reuse and regeneration of the coenzyme. Applications include NAD attachment to water-soluble polymers for use in synthetic and analytical reactors,[12] and NAD immobilization with water-insoluble polymers for use as the sorbent material for affinity chromatography.[13] Typically, the covalently modified NAD was used in an aqueous environment, whether or not the NAD was modified with a water soluble or insoluble polymer. Although it was covalently modified, the solubility of modified NAD was not investigated in organic solvents. We hypothesized that NAD covalently modified with a polymer will demonstrate solubility in an organic phase, in this case a fluorous solvent.

We have examined the covalent modification of NAD with a fluorinated polymer to enhance its solubility in a fluorous phase. Interestingly, molecules designed for solubility in fluorous solvents also demonstrate appreciable solubility in CO_2. Although CO_2 is a poor solvent for most compounds,[14] it has been shown to dissolve a number of fluorinated polymers at relatively ambient temperatures and moderate pressures.[15,16] In addition, attachment of fluorinated polymers have rendered other compounds soluble in CO_2.[17,18]

Herein, we report the synthesis of NAD with a covalently attached fluorinated polymer tail (FNAD). The solubility of FNAD was examined in both fluorous solvents and liquid CO_2. The ability for FNAD to act as a soluble coenzyme in enzyme-catalyzed oxidation/reduction reactions was explored in a fluorous solvent. To briefly summarize, issues such as how FNAD fared as a coenzyme when compared with native NAD, how the fluorinated polymer affected enzyme activity, and equilibrium of the recycling reaction system were explored. MALDI-TOF mass spectroscopy was used to substantiate that FNAD actually participated in a reaction catalyzed by a dehydrogenase. Finally, FNAD as a soluble coenzyme in liquid CO_2 was investigated.

2. Results and discussion

2.1. Synthesis of PFPE-modified NAD

FNAD (Fig. 3(b)) was synthesized in a four step process (Fig. 3(a)). NAD was brominated then reduced. The bromine was then displaced by hexanediamine (HDA) to

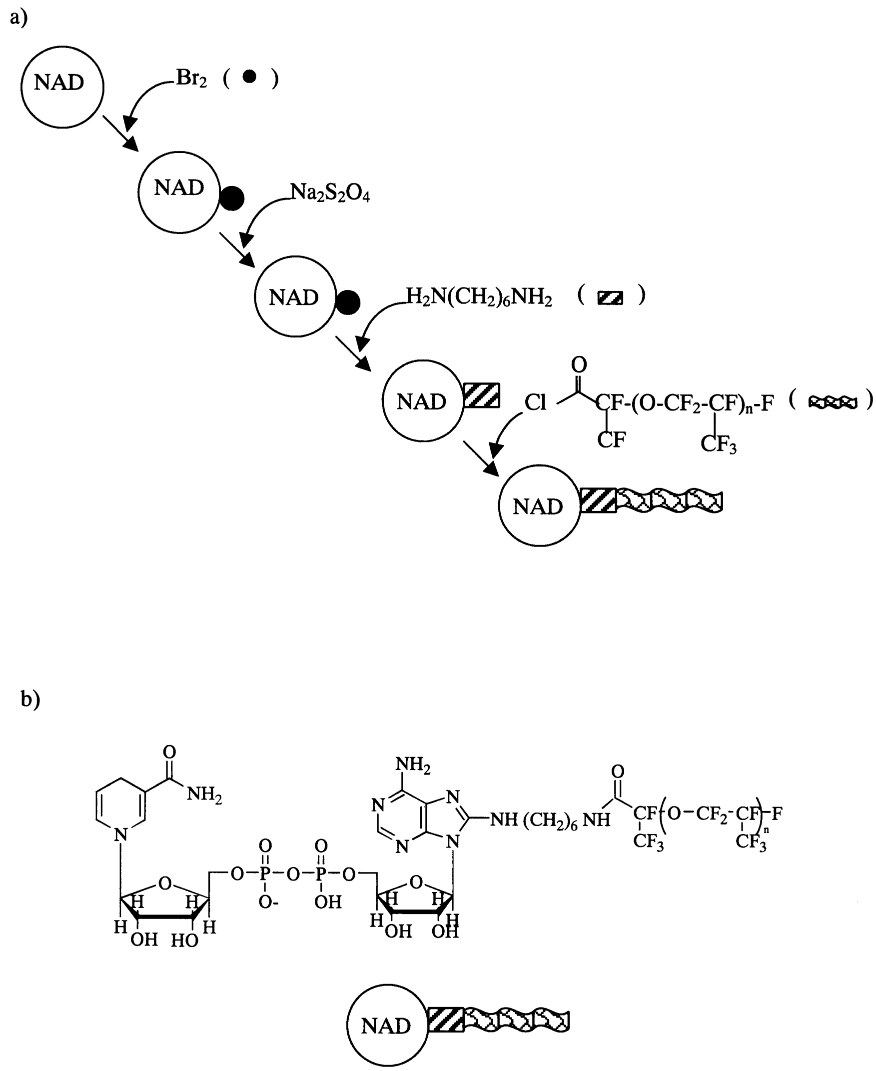

Figure 3. (a) Synthesis of FNAD, (b) structure of FNAD.

form NADH-HDA. Finally, a perfluoropolyether (PFPE) was appended to NADH-HDA through an amide linkage.

To NAD, which has a molecular weight of 664.4 g/mol, we attached a fluorinated polymer, PFPE, which has previously been shown to have high solubility in CO_2.[15] FNAD is hence an amphiphilic molecule with a hydrophilic head group and a fluorophilic/CO_2-philic tail. An important question was how large the molecular weight of the polymer must be in order to bring the large hydrophilic NAD into solution in a fluorous solvent or CO_2. For our purposes, a perfluoropolyether (PFPE) with a molecular weight of 2500 (~14 repeat units) was used to begin investigations.

Results showed that the 2500 MW PFPE was sufficiently large to solubilize FNAD in many fluorinated solvents. FNAD was soluble in methoxynonafluorobutane (HFE), 1,1,2-trifluorotrichloroethane, perfluorodimethylcyclohexane, perfluoromethylcyclohexane, perfluoroheptane, and perfluorohexane at levels of 200 mg/ml and above.

2.1.1. MALDI-TOF MS analysis. MALDI-TOF MS was used for two purposes in the analysis of FNAD: (1) to further confirm that a PFPE chain was attached to NAD, and (2) to demonstrate that FNAD acted as a coenzyme in the enzyme-catalyzed reaction. MALDI was effectively used to characterize FNAD, which is notable since MALDI has not been routinely applied to fluorinated polymers.[19] Most likely, the attached NAD head group facilitated analysis by this method. The matrix used for the analysis was 2,5-dihydroxybenzoic acid (DHB); however, *trans*-3-indoleacrylic acid (IAA) also yielded clean spectra with little background or noise. Different matrix to analyte molar ratios ($\sim 3\times10^4$:1–3×10^1:1) were investigated; however, there were no apparent changes in the spectra as a function of matrix/analyte ratio. Therefore, samples containing FNAD in HFE at a concentration of 15 mM were not further diluted before mixing with the matrix solution. No cationization agent was necessary, most likely due to the sites on the NAD molecule capable of ionization.

It is important to point out that while PFPE exhibits an average molecular weight of 2500 g/mol, it is a polydisperse material. With attached polymer, FNAD exhibits a molecular weight distribution of $923.4+166n$,

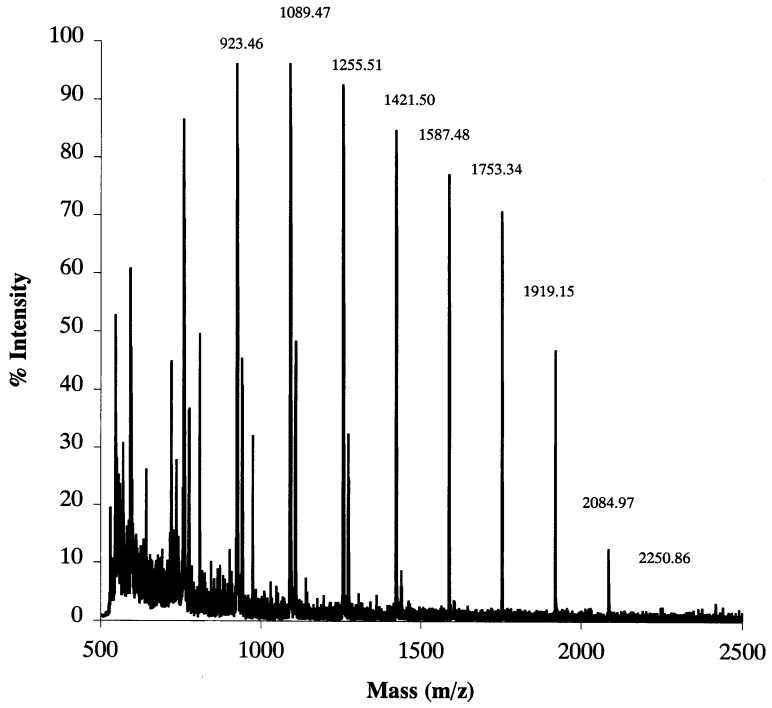

Figure 4. MALDI-TOF mass spectrum of FNAD.

where the 923.4 amu includes the NAD, the attached HDA, the amide linkage, and the end group of the polymer, and n is the number of repeat units in the polymer (Fig. 3(b)).

A MALDI-TOF mass spectrum clearly displays peaks of FNAD with attached polymers for n=1–7 repeat units (Fig. 4). Larger molecular weight species are not seen at the relative intensity of the lower molecular weight species; nonetheless, low intensity peaks representing molecular weights of 8–20 repeat units were also observed. Matrix concentration, sample concentration, laser intensity, and number of laser shots did not lead to higher molecular weight species being more visible. Higher molecular weight hydrophobic polymers are more difficult to analyze for several reasons. The polydispersity of the sample means that there are a mixture of oligomers in the sample; therefore, fewer molecules of each length of oligomer will be contained in the sample leading to lower sensitivity.[20,21] Furthermore, the matrix may not be efficient in solvating the longer chain polymers as it is with the low molecular weight species.[20,21] Lastly, the extraction and post-acceleration energies of the instrument may not be sufficient to maintain detector sensitivity for the large molecules.[20]

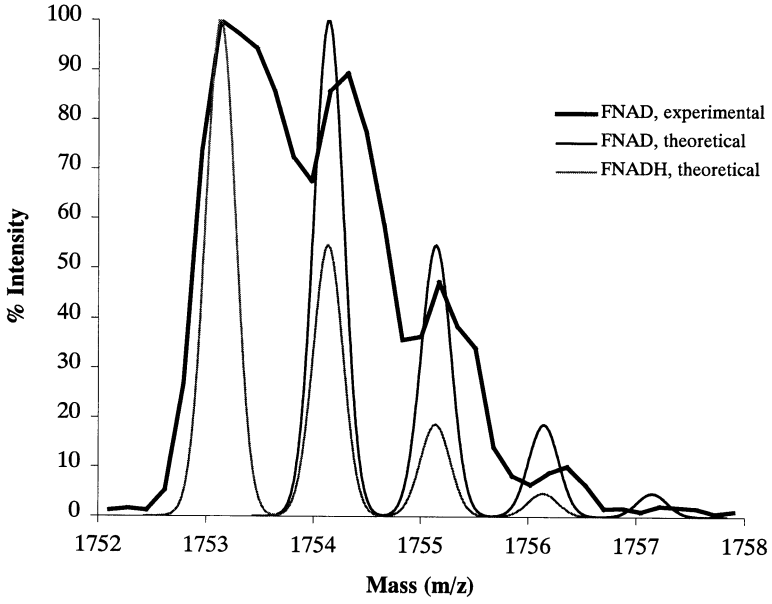

Figure 5. Overlapping mass spectra of the theoretical isotopic abundance of FNAD (n=5), the theoretical isotopic abundance of FNADH (n=5), and experimental FNAD (n=5).

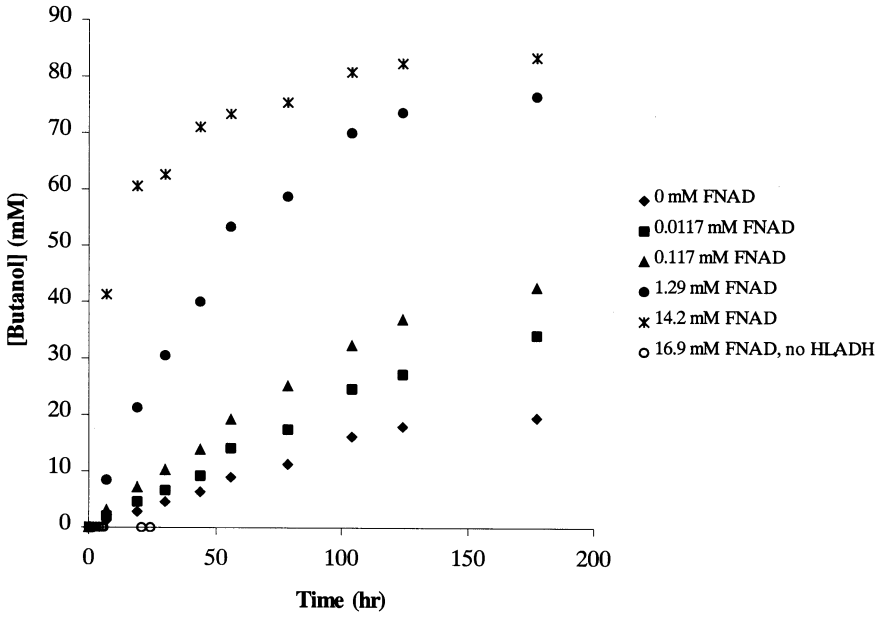

Figure 6. HLADH catalyzed reactions using FNAD as a soluble coenzyme in HFE.

Nevertheless, the spectrum clearly demonstrated that NAD was modified with perfluoropolyether molecules.

In the second reaction of the synthesis of FNAD, NAD was reduced to NADH; however, MALDI analysis showed that the sample after synthesis was actually a mixture of FNAD and FNADH. When FNAD accepts a hydride ion, its molecular weight increases by 1 amu; MALDI is indeed sensitive enough to distinguish a 1 amu difference. Further, the isotopic forms of the analyzed molecule can be detected by MALDI. The MALDI software can predict the theoretical isotopic abundance of a molecule based on the natural abundance ratios of the isotopes. Using FNAD with five repeat units attached ($C_{45}H_{40}N_9O_{20}P_2F_{35}$, MW=1753.4) as an example, the theoretical isotopic abundance spectrum for this molecule is shown in Fig. 5. Overlaid on that is the spectrum for the theoretical isotopic abundance of the reduced form, FNADH ($C_{45}H_{41}N_9O_{20}P_2F_{35}$, MW=1754.4). The actual spectrum for the sample FNAD is also displayed in Fig. 5. Isotopic abundance calculations for both FNAD and FNADH predict four peaks with decreasing intensity, (FNAD: 1753.4>1754.4>1755.4>1756.4 and FNADH: 1754.4>1755.4>1756.4>1757.4). The actual spectrum for FNAD does not have the same ratio of isotopic peaks as either theoretical spectrum; it is a combination of the two spectra as evidenced by the presence of five isotopic peaks. This qualitatively demonstrates that NADH-HDA is partially oxidized to NAD-HDA during the wash step with TFE. Unfortunately, quantitative amounts of each species cannot be ascertained from the MALDI data because it is unknown if one form is more readily desorbed by the matrix/laser combination.

2.2. FNAD as a soluble coenzyme in a fluorous solvent

2.2.1. HLADH activity using FNAD. Two major alterations to NAD have occurred that could influence coenzyme utility: first, the molecule was covalently modified, and second, the coenzyme was used in an unnatural environment. Either one of these issues alone could dramatically alter the ability of the coenzyme to function properly.

Activity studies in a fluorous solvent were performed in methoxy-nonafluorobutane (HFE) a solvent that is intended to replace ozone-depleting CFCs. HFE's benefits include zero ozone depletion potential, very low toxicity, and non-flammability. Horse liver alcohol dehydrogenase (HLADH), was used to catalyze both reactions as shown in Fig. 2. HLADH demonstrates broad substrate specificity, so it can be efficiently used to catalyze both the oxidation and reduction reactions. The forward reaction is the reduction of butyraldehyde (S1) to butanol (P1) using FNADH. The regeneration reaction is the oxidation of ethanol (S2) to acetaldehyde (P2) using FNAD. Ethanol is a common regenerative substrate due to its availability and low cost.

In this study, the functionality of FNAD was investigated by adding increasing amounts of coenzyme to reaction mixtures. Each sample contained increasing amounts of FNAD, from approximately 0 to 0.1 g, which corresponds to 0–15 mM, respectively. As can be seen from the graph (Fig. 6), increasing the amount of FNAD increased the rate of reaction until the butanol concentration reached a steady state.

Two control reactions were also performed. The sample that contained FNAD but no added HLADH was not active, as expected. However, the sample that did contain HLADH but no added FNAD showed substantial activity; therefore, the enzyme must contain residual bound NAD. A UV scan of the enzyme was performed to determine how much NAD was present; the scan showed that 0.1 mg of HLADH in H_2O had approximately a 46-fold molar excess of NAD associated with it. NAD is added during the purification of HLADH in order to stabilize the enzyme during the purification process[22]—if measures were used to remove the NAD, the HLADH would probably lose its activity.

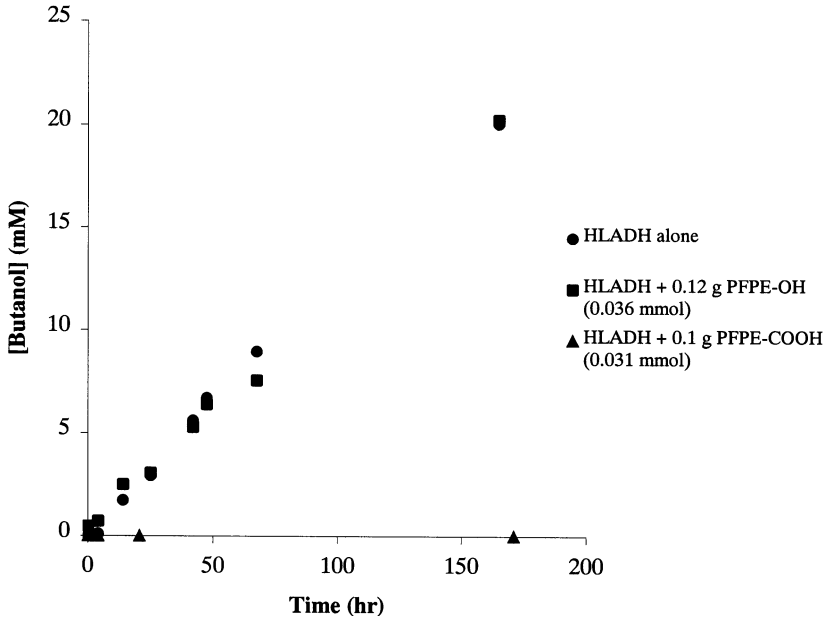

Figure 7. PFPE as a cosolvent.

Therefore, HLADH without added NAD is expected to have some native activity due to residual bound NAD.

2.2.2. Effect of PFPE on HLADH. HLADH without added FNAD demonstrated activity in HFE (Fig. 6), indicating that HLADH must have remaining NAD associated with it. This native activity leads one to question whether FNAD actually participated in the reaction. It is possible that the NAD head group did not actually participate in the oxidation/reduction reactions, but that the entire molecule itself or the PFPE tail acted as a cosolvent, and hence its presence in the reaction simply increased the enzyme activity in HFE. Solvent can have a tremendous effect on the activity of an enzyme. Binding of solvent molecules to the enzyme, partitioning of substrates and products between the solvent and enzyme, shifting of chemical equilibria, and mass transfer limitations are all known solvent effects on enzyme activity.[23] Polar cosolvents have been added to hydrophobic solvents to aid in solubilization of polar substrates.[24] Even water can act as a cosolvent when biocatalysis is performed in organic solvents.[25] DMSO and formamide have been used to activate several hydrolytic enzymes in anhydrous media, the activation most likely due to enhanced conformal flexibility of the enzymes in the anhydrous media.[26]

To test whether PFPE stimulated HLADH activity by acting as a cosolvent, the native activity of HLADH was compared with HLADH activity containing two different PFPE

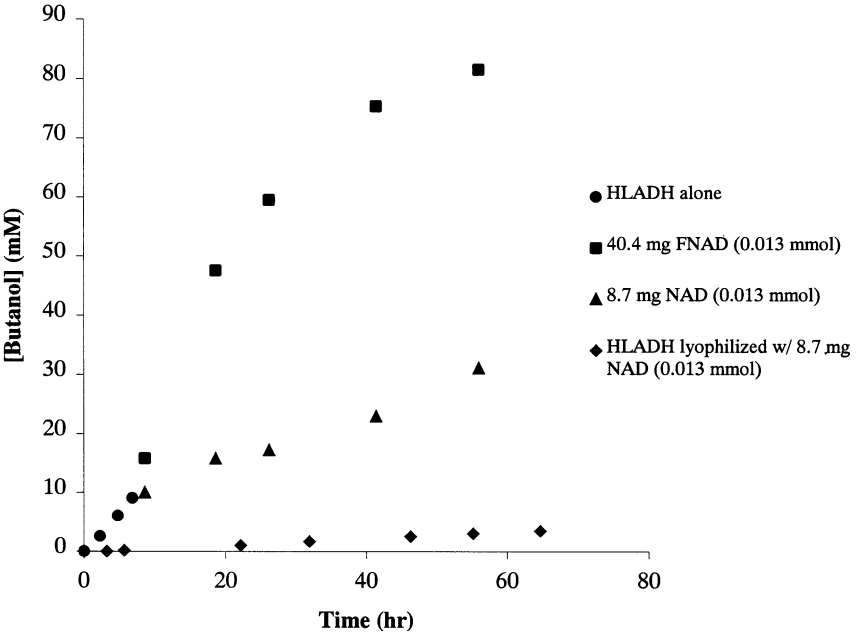

Figure 8. Comparison of FNAD and NAD.

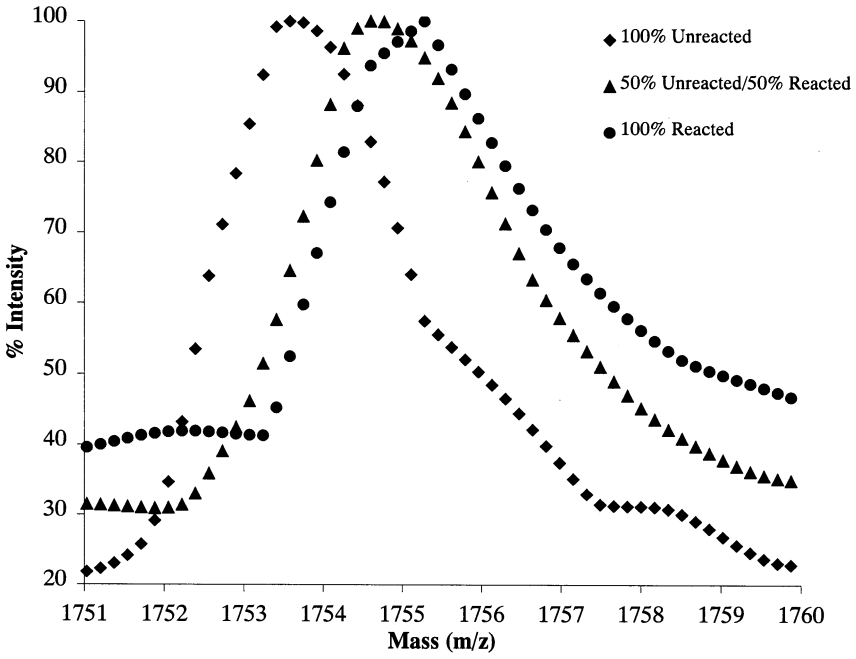

Figure 9. Overlapping MALDI-TOF mass spectra of unreacted and reacted FNAD (n=5).

polymers; one with a single terminal hydroxyl (PFPE-OH) and the other with a single terminal acid (PFPE-COOH). Addition of 0.12 g (0.036 mmol) PFPE-OH to the reaction mixture did not have any effect on the HLADH activity (Fig. 7). Conversely, supplementing the reaction mixture with 0.10 g (0.031 mmol) PFPE-COOH completely inactivated the enzyme. Most likely the acid group had an adverse effect on the enzyme, whereas the hydroxyl group was benign with respect to HLADH activity. In either case, though, it is obvious that PFPE itself did not aid the activity of HLADH by acting as a cosolvent in HFE.

2.2.3. FNAD versus NAD. Since the HLADH alone showed activity in HFE, one question is whether unmodified NAD (insoluble NAD) shows the same activity as the soluble FNAD. Consequently, the activity of NAD was compared with the same molar amount of FNAD using the same reaction scheme. Initially, HLADH (10 mg) was added to 250 mM of both butyraldehyde and ethanol in HFE (a total of 4 ml). The reaction proceeded for approximately 7 h, at which point the reaction was split into two samples. In one sample, 8.7 mg (0.013 mmol) of insoluble NAD were added. To the other sample, 40.4 mg of soluble FNAD were added at the same molar amount (0.013 mmol). The third sample contained 8.7 mg (0.013 mmol) of NAD that were directly lyophilized with the enzyme.

The reactions were allowed to proceed, monitoring the production of butanol. Evidently (Fig. 8), soluble FNAD promotes a faster reaction rate than either the insoluble NAD or the NAD directly lyophilized with the enzyme, indicating that solubilization of the coenzyme increases reaction rates. When NAD was added to the reaction as a solid it is possible that HLADH and NAD maintained their own hydration layers, but were not able to interact because they were both insoluble in the solvent. The HLADH demonstrated native activity due to residually bound NAD, but the addition of more NAD did not have any effect.

When NAD was directly lyophilized with HLADH, they shared a hydration layer. NAD was free to move about this layer to participate in the reactions; however, the activity was much less that when the NAD was separate from the HLADH. A possible explanation was that too much NAD was present leading to mass transfer limitations. A lyophilized enzyme particle is a porous structure and a substrate molecule has to negotiate the pores to reach the active site.[27] By lyophilizing HLADH with NAD, increased internal diffusional limitations within the lyophilized enzyme/NAD complex most likely hindered the substrate from reaching the active site of the enzyme, thereby decreasing activity. Although, Yang demonstrated that yeast alcohol dehydrogenase (YADH) activity increased with an increase in NAD/YADH ratio,[10] the maximum molar ratio of NAD/YADH was 4.25. The NAD/HLADH herein was about 210 (not including the native, residually bound NAD). NAD was in great molar excess, which most likely just increased the solid mass, but did not lead to efficient enzyme activity.

2.2.4. MALDI-TOF analysis of FNAD. MALDI-TOF mass spectroscopy was used to confirm the structure of FNAD and that FNAD was actually a mixture of both the oxidized and reduced forms of the coenzyme. Moreover, MALDI was used to verify that the FNAD molecule, not just native bound NAD, participated in the reactions.

FNAD was used in the following reaction:

$$CH_3CH_2OH + FNAD \overset{HLADH}{\rightleftharpoons} CH_3CHO + FNADH + H^+$$

This reaction did not include a regeneration reaction. Different MALDI samples were prepared by spiking

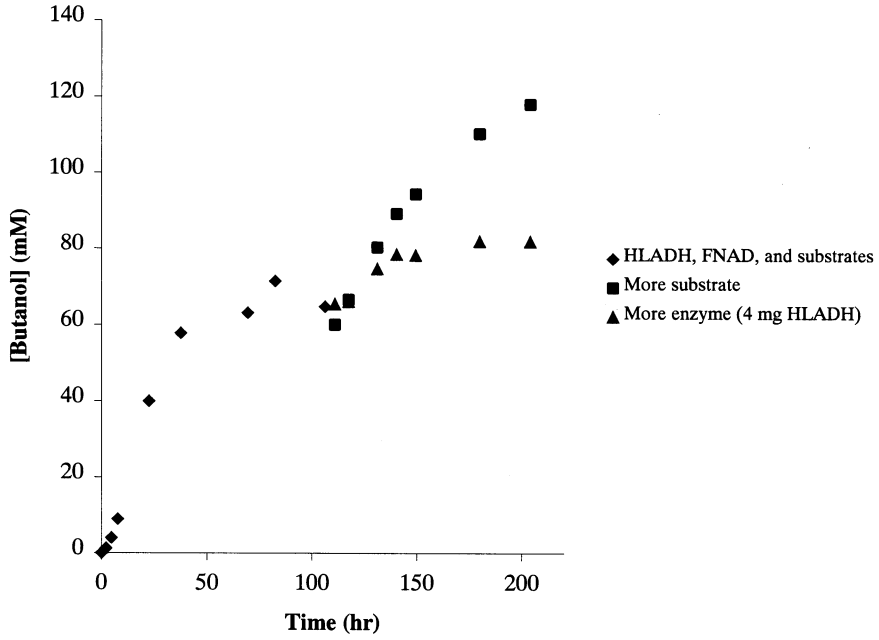

Figure 10. Equilibrium of HLADH catalyzed reactions using FNAD as a soluble coenzyme.

'unreacted FNAD' with increasing amount of 'reacted FNAD'. It would be expected that peaks on the MALDI spectra would show an increase in the molecular weight by 1 amu between the unreacted FNAD and the reacted FNAD.

Indeed, the MALDI spectra show that peaks of the reacted FNAD have a greater molecular weight than the peaks of the unreacted FNAD. Fig. 9 shows the peaks of FNAD (n=5) of the spiked samples superimposed on each other. The peak molecular weight shifts to a higher molecular weight with increasing amount of the reacted FNAD in the sample. The same result was seen with six repeat units. Therefore, more FNADH was produced during the reaction indicating that FNAD did participate in the reactions.

2.2.5. Equilibrium. As shown in Fig. 6, the production of butanol slowed until there was no further formation beyond a certain time. Two explanations could account for this phenomenon: (1) either the enzyme was no longer active (enzyme death) or (2) the system reached equilibrium.

There are two methods to distinguish whether the system reached a steady state due to enzyme death or equilibrium:

1. At the apparent equilibrium point, more enzyme can be added to the reaction mixture. Because catalysts do not affect equilibrium, if the system were at equilibrium, adding more enzyme should not change the composition of the reaction mixture.
2. At the apparent equilibrium point, more substrate can be added. Adding or removing a reactant or product will disturb the equilibrium. If the system were at equilibrium, adding more substrate will shift the equilibrium to produce more butanol.

The two options were investigated to determine if the system had reached equilibrium or if enzyme death had occurred (Fig. 10). In a 10 ml glass vial, 250 mM butyraldehyde and ethanol, 3 mM FNAD, and 10 mg HLADH in a total of 4 ml HFE were added. The reaction was shaken at 250 rpm and 30°C, and the production of butanol was followed with time. When it appeared that no further butanol was being produced, the reaction mixture was split into two, with 2 ml in each vial. To the first vial, 4 mg of fresh HLADH was added. To the second vial, 250 mM of both butyraldehyde and ethanol were added. The vials were further shaken at 250 rpm and 30°C and the production of butanol was followed with time.

The reaction that received the additional 4 mg of HLADH did not see a great increase in butanol concentration, reaching a final butanol concentration of 80 mM (Fig. 10). However, the reaction that received 250 mM of both butyraldehyde and ethanol showed an increase in butanol concentration, reaching 120 mM butanol. Apparently the system was at equilibrium. Adding more substrate shifted the equilibrium position of the reaction resulting in the production of more butanol. The equilibrium position for the reaction starting with 250 mM butyraldehyde and ethanol contains 80 mM butanol and therefore 170 mM butyraldehyde, which agrees with the data in Fig. 6.

Acetaldehyde (P2) is volatile (bp=21°C), hence it would be expected to evaporate during the reaction. However, acetaldehyde apparently remained in the reaction mixture, as seen on the chromatograms generated from GC and because the system was able to reach equilibrium. Several possible reasons exist to explain why acetaldehyde did not simply evaporate: (1) NAD has been shown to form adducts with substrates such as butyraldehyde and acetaldehyde;[28] (2) acetaldehyde condenses with NADH to give 1,2-dihydro-2-ethylidenenicatinamide adenine dinucleotide;[29] and (3) acetaldehyde interacts with proteins.[30]

To drive the reactions forward, acetaldehyde must be

Table 1. TON of FNAD HFE (177 h)

FNAD (mM)	TON	Butanol produced (mM)	[FNAD]/[E]
HFE (177 h)			
0.012	1199	14.4	0.4
0.12	192	23.1	3.8
1.3	44	56.9	41.6
14	5	63.7	454.4
CO_2 (57 h)			
4	9	34.5	253.2

removed from the reaction mixture, which may be accomplished by two simple methods. One technique is to bubble nitrogen in the reaction mixture and trap it in a vessel containing semicarbazide to form acetaldehyde semicarbazone.[31] Another method is to oxidize acetaldehyde to acetate using the enzyme aldehyde dehydrogenase (AldDH), which also generates a second equivalent of reduced coenzyme.[32,33] Acetate does not harm enzymes or coenzymes nor does it complicate product isolation.

With the removal of acetaldehyde, the reaction would be pushed to completion, thereby eliminating the need of separation of the other substrates from the product of interest. Furthermore, enzyme may be recovered by simple filtration and FNAD by a fluorous separation technique.[8]

2.2.6. Turnover number. Conventional TON's for aqueous enzymatic reactions do not exceed 2000,[34,35] whereas turnovers have reached 2,000,000 in organic solvents.[36] The TONs of FNAD were determined from the reactions shown in Fig. 6, which took place over the time course of 177 h. The data was corrected by subtracting the native activity of HLADH in order to distinguish the activity due to FNAD. Table 1 reports the TON, the concentration of butanol produced in each reaction, and the ratio of FNAD to enzyme in the sample. Although 14 mM FNAD produced the most butanol in the shortest time, the TON was very low, indicating that the FNAD was not being used efficiently. When too much FNAD was added, the turnover number was low because the enzyme was saturated with the coenzyme. FNAD thus acted more like a cosubstrate than a recycling coenzyme at high concentrations. Nonetheless, the reactions at higher FNAD concentrations (14 mm and 1.29 mM) had reached equilibrium so it is likely that the TON for these two samples could be much higher. On the other hand, the sample with the lowest concentration of FNAD (0.012 mM) had the highest TON but the lowest production of butanol in the time period. The implication is that TON and activity must be optimized to achieve the most efficient reactions.

2.2.7. Side reaction. *n*-Butyraldehyde is known to undergo base-catalyzed aldol reactions.[37] Both enzymes[38,39] and NAD have basic moieties that are able to catalyze this reaction, resulting in a competing reaction for butyraldehyde. The side reaction is accelerated by the presence of contaminating HDA in FNAD and by degradation products in butyraldehyde; therefore, care must be taken to insure the purity of FNAD and to use fresh and properly stored butyraldehyde. Nevertheless, this side reaction was not completely eliminated.

2.3. FNAD as a soluble coenzyme in CO_2

2.3.1. Solubility studies in CO_2. A cloud point curve was generated to determine the solubility of FNAD in CO_2 (Fig. 11). The range of concentrations was approximately 3–5 mM, which corresponds to 1.2–2 wt%, respectively, and typically, the concentration one would expect to use in a reaction. As can be seen from the graph, the cloud point pressures are relatively constant over the weight percent range of interest, the cloud points being between 1200 and 1400 psi. The goal is to perform enzyme catalyzed oxidation/reduction reactions in CO_2 with a CO_2-soluble coenzyme; therefore, the operating pressure should be set where the FNAD exists as one phase with the CO_2, or at a pressure above the cloud point curve.

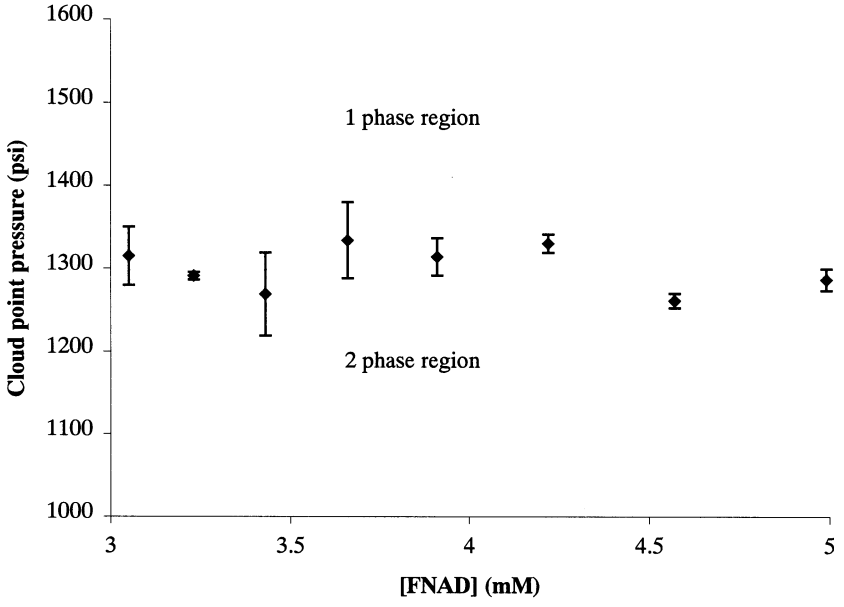

Figure 11. Cloud point curve of FNAD in CO_2 (T=21°C).

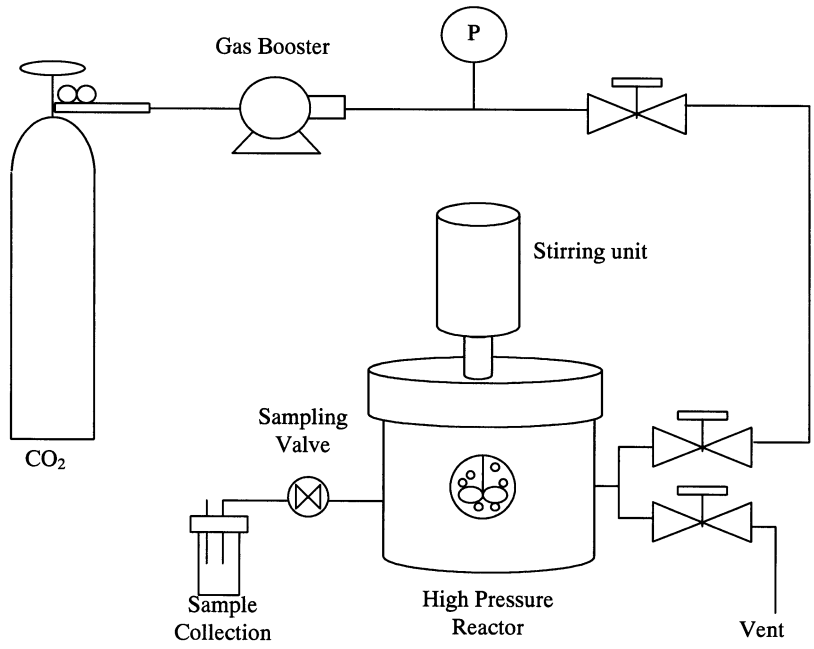

Figure 12. High-pressure CO_2 reactor.

Many different compounds have been covalently modified with CO_2–philic functionalities to enhance their solubility in CO_2. Examples include surfactants,[15] a biological molecule,[17] and metal chelating agents.[18] In each case, the length of the CO_2–philic tail and the degree of modification of the molecule all affected the solubility of the molecule in CO_2.[15,17,18] By increasing the size of the tail, the number of CO_2–philic interactions increases, thereby increasing solubility. But soon there is a point where increasing the tail further just leads to an increase in the entropy of mixing, which decreases the solubility. However, one tail with a molecular weight of 2500 was sufficient to solubilize NAD in CO_2.

In addition to determining the solubility of FNAD in CO_2, it was also necessary to determine if all of the substrates and products were soluble in liquid CO_2 at the required concentrations (250 mM). Solubility of the product was not as important, as an insoluble product would drive the reaction forward. The cloud points of butanol and butyraldehyde were generated over the concentration range of 302.5–195.8 and 298–193 mM, respectively, which was the substrate concentration range used in the reactions in HFE. Both butanol and butyraldehyde were soluble in CO_2 at pressures greater than 1800 psi.[40] Ethanol and acetaldehyde were also miscible with CO_2 under those conditions.

2.3.2. Activity of HLADH using FNAD in high pressure CO_2. Fig. 12 shows the schematic of the high pressure

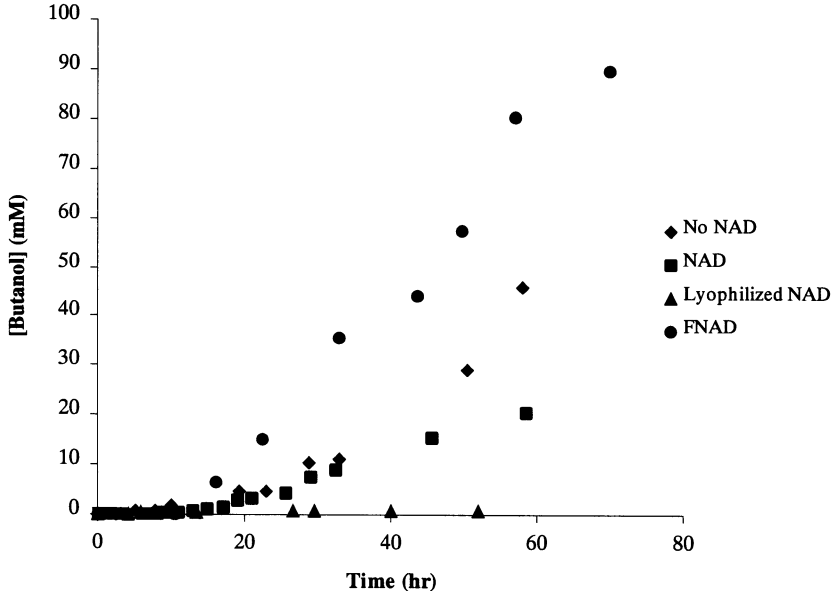

Figure 13. HLADH catalyzed reactions using FNAD as a soluble coenzyme in CO_2 (T=21°C, P=2600 psi).

reactor used to study the activity in CO_2. HLADH activity in liquid CO_2 at 2600 psi and room temperature was investigated using FNAD as a soluble coenzyme (Fig. 13). Four different reactions were run: (1) HLADH alone; (2) HLADH and 0.4 g (0.125 mmol) FNAD; (3) HLADH and 83 mg (0.125 mM) NAD added separately; and (4) HLADH lyophilized with 83 mg (0.125 mM) NAD. The FNAD and NAD were provided in equimolar amounts. The concentration of FNAD was 4 mM, which at 2600 psi is a single phase mixture (Fig. 11).

HLADH in each case was active in liquid CO_2. To date, no other studies on purified alcohol dehydrogenase activity in CO_2 have been reported. The only investigation of alcohol dehydrogenase activity in CO_2 has been performed using whole cells, rather than isolated enzyme.[41] The resting cells of a fungus *Geotrichum candidum* IFO 5767 were effectively employed for the reduction of *o*-fluoroacetophenone to (*S*)-1-(*o*-fluorophenyl)ethanol in supercritical CO_2 (35°C and 10 MPa).[41] Other substrates such as acetophenone, acetophenone derivatives, benzyl acetone, and cyclohexanone were all reduced by the cells. Since whole cells were used, neither additional NAD nor a regenerating substrate was provided. However, the cells were suspended in water containing propan-2-ol before immobilization on a water-absorbing polymer.

As shown in Fig. 13, the activity of each reaction in CO_2 followed the same trend as the reactions performed in the fluorous solvent. The sample with the soluble coenzyme demonstrated the highest activity. The sample with enzyme alone and the sample with added NAD showed similar rates of butanol production, until 45 h, when the rates deviated. The enzyme alone continued to produce butanol, whereas the activity of the enzyme with added NAD leveled off. The reason for this is unknown. Finally, the HLADH that was lyophilized with NAD produced very little butanol (1.2 mM). Once again, the reasons for the trends in activity seen in HFE also apply here. The high activity for HLADH with FNAD was due to the solubility of the coenzyme; the low activity for HLADH lyophilized with NAD was likely due to mass transfer limitations; and NAD added separately from the enzyme did not change native HLADH activity because the enzyme and NAD remained separate during the reaction.

HLADH without any added coenzyme or modified coenzyme exhibits high activity, more so than the same sample in HFE. Although the reactions in CO_2 and HFE were not performed under exactly the same conditions, a comparison can be made between them to illustrate the high activity of HLADH in CO_2. As shown in Fig. 6, the sample of HLADH with no added coenzyme or derivative of the coenzyme produced about 8.6 mM butanol in 56 h. However, the sample in CO_2 resulted in approximately 45.9 mM butanol being produced in 58 h (Fig. 13). Five times more butanol is produced in CO_2 than in HFE within the same time period. The reason for the difference may be that the diffusional limitations between solid enzyme and the native-bound NAD that exist in liquid solvents are alleviated somewhat in near-critical CO_2 due to the benefits of gas-like diffusivities and viscosities. This may indicate that a soluble version of the coenzyme is not as useful in a supercritical or near-supercritical fluid as it is in a liquid solvent due to the inherent properties of supercritical and near-supercritical fluids. Yet despite the high activity of HLADH in CO_2, the addition of soluble coenzyme still does lead to higher production of butanol.

The sample with FNAD was run an extra 10 h longer than the other reactions. It appeared that the production of butanol may have leveled off at that point, indicating that the reaction may have reached equilibrium. The concentration was 89 mM, slightly higher than the equilibrium concentration in HFE, which was 80 mM; however, the difference most likely is due to the error in sampling from the high-pressure reactor. To confirm that the system actually does reach equilibrium, the reaction would have to be run for longer times.

The TON of FNAD in CO_2 was calculated in the same method as the samples in HFE. The data was corrected by subtracting the native activity of HLADH in order to distinguish the activity due to FNAD. The TON of FNAD in CO_2 at approximately 57 h is 9. The results are reported in Table 1.

3. Conclusions

A fluorofunctional NAD (FNAD) was synthesized and found to be soluble in many fluorous solvents. FNAD showed great utility as a coenzyme. Solubilization of the coenzyme in HFE resulted in faster reactions when HLADH was used to catalyze oxidation/reduction reactions, much faster that the same molar amount of NAD. MALDI-TOF MS data confirmed that FNAD participated in the reduction reaction. FNAD at concentrations between 3 and 5 mM were also soluble in CO_2 at pressures between 1200 and 1400 psi. An oxidation/reduction reaction was catalyzed by HLADH in CO_2 at 2600 psi and room temperature (well above the cloud points of the substrates and FNAD) using FNAD as a soluble coenzyme. Like in HFE, solubilization of the coenzyme led to faster reaction rates than reactions performed with the same molar amount of insoluble NAD. In addition, relatively the same amount of butanol was produced in CO_2 over the same time as in HFE.

4. Experimental

4.1. Materials

Nicatinamide adenine dinucleotide from Baker's Yeast and alcohol dehydrogenase from horse liver (HLADH) were purchased from Sigma Chemical Company. Krytox® 157 FSL (Dupont), a perfluoropolyether (PFPE) with a terminal COOH functionality (MW 2500), was procured from Miller Stephenson. Methoxynonafluorobutane (HFE) was obtained from 3M. Carbon dioxide at 99.9% purity was obtained from Praxair (3.0 grade). All other chemicals were purchased from Aldrich Chemical Company and used as received unless otherwise stated. Spectra cellulose ester dialysis tubing and Whatman filters (size 4) were obtained from Fisher Scientific.

4.2. Bromination of NAD

NAD was brominated at the C-8 position of adenine by a modification of the method of Lee and Kaplan.[42] Typically, 2.5 g of NAD were dissolved in 30 ml of 0.5 M sodium acetate buffer, pH 4.5. To this solution was added 1 ml bromine and the mixture stirred at room temperature for 1 h. The unreacted bromine was removed by extraction with an equal volume of carbon tetrachloride until neither phase changed color (approximately 7×). The solution was dialyzed against water in 500 MW cut-off cellulose ester dialysis tubing (Spectra) for 8 h (or overnight). The resulting solution was lyophilized (Labconco Freezedrier) for 48 h. Bromination of NAD was confirmed by ^{1}H NMR (Bruker) using deuterated water as the solvent.

The bromination of NAD occurred on the C-8 position on the adenine group of NAD. This reaction was confirmed using ^{1}H NMR. Before bromination, NAD had five protons attached to aromatic groups, four on nicatinamide (δ=9.45, 9.31, 9.28, and 8.42 ppm) and one on adenine (δ=8.31 ppm). The position of the resonance frequency at position C-8 also occurs in this range (δ=8.62 ppm), resulting in six peaks on the ^{1}H NMR spectrum between 8 and 10 ppm. After bromination, only five peaks are present in the region (nicatinamide protons: δ=9.35, 9.16, 8.86, and 8.45 ppm, adenine proton: δ=8.18 ppm) because the proton at position C-8 was replaced with bromine. Reaction was complete upon disappearance of the peak representing the proton at the C-8 position on adenine (δ=8.62 ppm).

4.3. Reduction of NAD-Br

NADH-Br has been found to be more thermally stable than NAD[43] and hence NAD-Br was chemically reduced to NADH-Br using sodium dithionite. NAD-Br (0.5 g) was dissolved in 1.3% sodium bicarbonate buffer, which had been deaerated first by bubbling with argon or nitrogen for 15 min. A two-fold molar excess of sodium dithionite (0.25 g) was added, and the solution shaken for 1 h at 25°C. The sodium dithionite remaining in the reaction mixture was oxidized by bubbling oxygen through the solution for 15 min. The NADH-Br was recovered by precipitating with 10× the volume of acetone, the supernatant was removed, and the product was air dried. The product was resuspended in 10 mM sodium phosphate buffer, pH 7.8, followed by lyophilization for 48 h. Reduction was verified using UV spectroscopy (Perkin–Elmer Lambda 2) by the increase in absorbance at 340 nm. The molar adsorption coefficients of NAD at 260 and 340 nm are 18,600 and 6650 mol/l cm, respectively. Once the reduction was complete, the ratio of the absorbance of 260–340 nm was approximately 3.[42]

4.4. Reaction of hexanediamine and NADH-Br

Hexanediamine was used to displace the bromine at the C-8 position of adenine. Typically, NADH-Br (2 g) was dissolved in 20 ml DMSO. The solution was stirred and heated to 80°C, and the system was flushed and blanketed with argon or nitrogen. A 20× molar excess of hexanediamine (HDA) in 20 ml DMSO was injected into the solution by a glass syringe. The reaction mixture was stirred for 2 h at 80°C, after which, the reaction was cooled to room temperature and stirred for an additional 6 h.

The reaction was confirmed using UV spectroscopy monitoring a peak maximum shift from 264 to 277 nm.[42] The product (NADH-HDA) was precipitated from solution using a 10× volume of acetone. The product was recovered by centrifuging (Sorvall Superspeed RC2-B Automatic Refrigerated Centrifuge) at 5000 rpm for 5 min to pellet the precipitate. The supernatant was removed and the product was washed in 2,2,2-trifluoroethanol, then filtered (Whatman filters). The filtrate was passed through a gel filtration column (Sephadex G-10) to remove unreacted HDA. The solvent was removed under vacuum.

4.5. Reaction of PFPE acid chloride with NADH-HAD

PFPE acid chloride was prepared as in Yazdi and Beckman.[18] The product was confirmed by a shift of the IR carbonyl peak from 1776 to 1806 cm^{-1} (Genesis-II IR spectrophotometer, Mattson).

In the reaction of NADH-HDA with PFPE acid chloride, the molar ratio of NADH-HDA to PFPE acid chloride was 1.2:1. NADH-HDA and an equimolar amount of triethylamine (TEA) in 50 ml TFE were added to a three neck round bottom flask. In an addition funnel, PFPE acid chloride was dissolved in 20 ml 1,1,2- trifluorotrichloroethane. The system was flushed and blanketed with argon or nitrogen. The PFPE acid chloride solution was added dropwise to the NADH-HDA solution. The mixture was stirred 12–15 h at 30°C. The solvent was removed under reduced pressure. The product was washed with 1,1,2-trifluorotrichloroethane, filtered, and the solvent removed under reduced pressure. The product was resuspended in 20 ml perfluorodimethylcyclohexane and placed in a separatory funnel overnight. The bottom layer was removed and the solvent removed under reduced pressure. IR spectroscopy was used to substantiate the formation of the amide linkage between the free amine on hexanediamine and the PFPE acid chloride. After the reaction, the IR peak at 1806 cm^{-1} disappeared and a peak at 1714 cm^{-1} indicative of a fluorinated amide linkage was present.

The attachment of the spacer molecule to NAD requires an excess of the diamine. If NAD-HDA is not free of HDA, the diamine will react with the PFPE-acid chloride instead of the modified NAD. When HDA reacts with PFPE acid chloride, an IR peak for the amide linkage between the diamine and PFPE-acid chloride appears very close to, but at a somewhat lower wavenumber (1704–1708 cm^{-1}) than that of FNAD (1714 cm^{-1}).

Another important peak on the IR spectrum that confirms the presence of FNAD versus HDA-PFPE is around 1668 cm^{-1}, which correspond to the amide linkage on the nicatamide group. This peak is sometimes not separate from the peak at 1714 cm^{-1}, but a large shoulder will be apparent. This peak is absent when the excess diamine reacts with PFPE-acid chloride instead of the NAD.

4.6. MALDI-TOF MS analysis

MALDI-TOF MS analysis was performed on both an unreacted FNAD sample and a reacted FNAD sample. To prepare the unreacted sample, FNAD at a concentration of 15 mM was prepared in a solution of 250 mM ethanol and 250 mM butyraldehyde in HFE. Samples were prepared for MALDI analysis by mixing the matrix 2,5-dihydroxybenzoic acid (DHB, 30 mg/ml in tetrahydrofuran) and 15 mM FNAD solution in a 5:1 ratio, respectively. Two microliters were spotted on the sample plate and the spot was allowed to air-dry in order to evaporate the solvent.

To prepare the reacted sample, 5 mg of HLADH were added to a solution of 15 mM FNAD and 250 mM ethanol in HFE in a 4 ml glass vial. The reaction mixture was shaken at 250 rpm for 48 h at 30°C, after which the enzyme was removed from the solution by filtration (Acrodisc CR PTFE 0.45 μm syringe filters). This sample was labeled reacted FNAD. A series of different samples were prepared by spiking the unreacted FNAD with increasing amounts of reacted FNAD. Spiked samples were prepared for MALDI analysis by mixing with 30 mg/ml DHB in tetrahydrofuran in a 5:1 matrix/FNAD ratio. Two microliters were spotted on the sample plate, followed by air-drying.

MALDI analysis was performed using a Perseptive Biosystems Voyager DE STR Biospectrometry Workstation 4087. The spectrometer was operated in linear mode, 20 kV accelerating voltage, 1997 laser intensity, 50 shots/spectrum. Data Explorer Software version 3.5 was used to analyze the results. Data was saved as ASCII files, and converted in Excel documents for graphical presentation.

4.7. Enzyme preparation

HLADH from Sigma was obtained as a dry, lyophilized powder. The enzyme was resuspended in 10 mM sodium phosphate buffer, pH 7.8, to a final concentration of 5 mg/ml. The enzyme solution was aliquotted into 1 ml portions in 15 ml conical tubes (Falcon). The enzyme solutions were snap frozen in liquid nitrogen and lyophilized for 48 h. The enzyme was stored at −20°C until use.

4.8. Activity studies in HFE

A typical reaction contained FNAD and 5 mg HLADH in a solution of 250 mM butyraldehyde and ethanol in HFE in a 4 ml screw cap glass vial (Wheaton). The total reaction volume was 2 ml. The vials were shaken at 250 rpm and 30°C. The reaction mixtures were analyzed for the product, butanol, with time using a FID-GC (Perkin–Elmer). The solid phase in the capillary column was cross-linked-methyl siloxane and the column dimensions were 30 m×0.55 mm×0.88 μm film thickness. A calibration curve was prepared for butanol in HFE at concentrations from 0 to 50 mM. The calibration curve was used to convert the peak areas from the GC to the corresponding concentrations of butanol.

4.9. Solubility studies in CO_2

Solubility studies were performed at room temperature in a variable volume high-pressure view cell. A known amount of sample and CO_2 were added to the view cell in order to get a specific weight percent of FNAD in CO_2. The pressure of the CO_2 in the system was increased using a gas booster and syringe pump until the FNAD in the CO_2 exhibited one phase (FNAD was soluble in CO_2). The volume of the cell was then expanded using a second syringe pump to decrease the pressure until the solution inside the cell became turbid. At that pressure, the FNAD was no longer soluble in the CO_2 and it was labeled the cloud point pressure. More CO_2 was then added to the system to dilute the sample and the cloud point pressure was determined for the new weight percent of FNAD in CO_2. A cloud point curve was generated by determining the cloud point pressures at different weight percents of FNAD in CO_2.

4.10. Activity studies in CO_2

The high pressure reactor used to study the activity in CO_2 had a volume of 30 ml (Fig. 12). Samples were added through the top of the reactor and the lid screwed in place. CO_2 was added and the pressure in the system increased using the gas booster. The mixture within the reactor was continuously stirred (Parr) at room temperature. Samples were taken by opening a 200 μl sample port and bubbling the reaction mixture through a solvent, toluene. The samples were analyzed by GC. Due to the error associated with sampling, a calibration curve of the ratio of the area of the butanol peak to the area of the butyraldehyde peak versus the known butanol concentration inside of the reactor was created.

To study activity, 250 mM butyraldehyde, 250 mM ethanol, and 38 mg HLADH were added to the reactor with either (1) no added coenzyme, (2) NAD, or (3) FNAD. CO_2 was added at room temperature and 2600 psi (well above the cloud points of the substrates and FNAD). The reaction mixture was sampled at regular intervals in order to monitor the production of butanol.

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