One may also anticipate that since these reagents provide a straightforward method of attaching a metal chelate function to a substrate, there may be many other applications unrelated to NMR, such as the functionalization of surfaces or polymers.

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Registry No. $C_6H_5B(pz)_3Na$, 80583-77-3; $4\text{-}BrC_6H_4B(pz)_3Na$, 80593-37-9; $4\text{-}LiC_6H_4B(pz)_3Na$, 80583-78-4; $(4\text{-}BrC_6H_4B(pz)_3)_2Co$, 80583-79-5; $(C_6H_5B(pz)_3)(4\text{-}BrC_6H_4B(pz)_3)Co$, 80583-80-8; $(4\text{-}n\text{-}BuC_6H_4B(pz)_3)_2Co$, 80593-38-0; $(4\text{-}HOOCC_6H_4B(pz)_3)_2Co$, 80583-81-9; $(4\text{-}CH_3OOCC_6H_4B(pz)_3)_2Co$, 80583-82-0; $(4\text{-}LiC_6H_4B(pz)_3)_2Co$, 80583-83-1; $PhB(OH)_2$, 98-80-6; $4\text{-}BrPhB(OH)_2$, 5467-74-3; pyrazole, 288-13-1.

Hybrid Organometallic/Enzymatic Catalyst Systems: Regeneration of NADH Using Dihydrogen[1]

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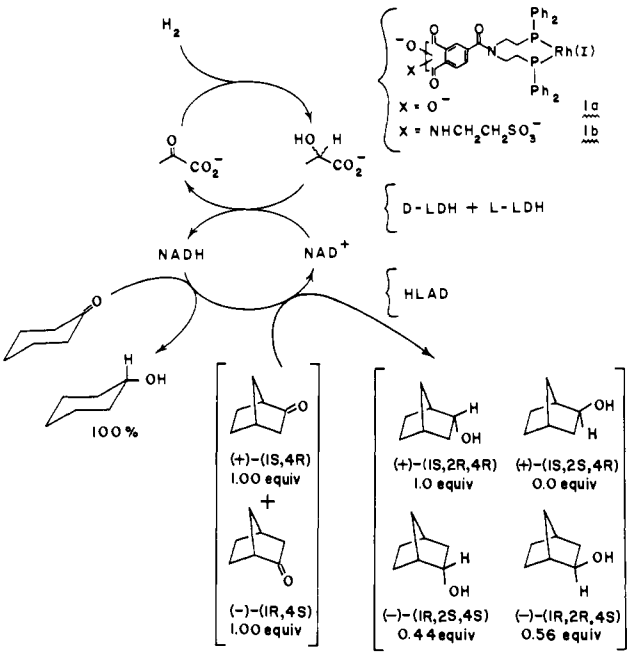
Abstract: A mixture of a water-soluble bis(phosphine)rhodium complex and D- and L-lactate dehydrogenase catalyzes the reduction of nicotinamide adenine nucleotide (NAD^+) to NADH by H_2 in an aqueous solution containing pyruvate (Scheme I). Coupling of this system to the asymmetric reduction of 2-norbornanone using horse liver alcohol dehydrogenase is demonstrated.

Organometallic and enzymatic catalysts generally exhibit their most characteristic activities on different classes of reactants: organometallic catalysts are especially effective with nonpolar substances (e.g., H_2, CO, and olefins), while enzymatic catalysts are most effective with polar, polyfunctional materials (e.g., carbohydrates, derivatives of acids, and biopolymers). Combinations of organometallic and enzymatic components which integrate these two types of substrate selectivities may have uncommon and useful catalytic activities. Here we demonstrate the operation of a hybrid system involving the combination of a water-soluble bis(phosphine)rhodium complex with two enzymes, whose overall action is to catalyze the reduction of NAD^+ to NADH by H_2.[2] The operation of this system and illustrations of its application to enzyme-catalyzed reduction reactions requiring NADH regeneration are summarized in Scheme I. The regeneration of the nicotinamide cofactors (NAD(P)-NAD(P)H) has been the subject of previous research in our laboratories. Several procedures are presently available;[3-8] the evaluation of their relative merits has not been completed.

Scheme I. Reductions of Carbonyl Compounds Using a Hybrid Organometallic-Enzymatic Catalyst System[a]



[a] D(L)-LDH = D(L)-lactate dehydrogenase; HLADH = horse liver alcohol dehydrogenase.

Results

Reduction of (±)-2-Norbornanone. Asymmetric reduction of (±)-2-norbornanone was carried out in a system containing racemic sodium lactate, NAD^+, coimmobilized horse liver alcohol dehydrogenase (HLAD), D- and L-lactate dehydrogenase (D- and L-LDH), the bis(phosphine)rhodium complex **1a** or **1b** (originally as $L_2Rh^I(norbornadiene)^+CF_3SO_3^-$), and dihydrogen at 40 psi. After 3 days, 50% reduction had occurred and no further reaction was observed. The residual activities of HLAD and D- and L-LDH at this time were respectively 80%, 85%, and 82% of the original immobilized activities. The aggregate residual activity of NAD^+ and NADH was 80% of the original value. The reaction was continued by introducing additional rhodium complex into the

(1) Supported by the National Institues of Health (Grant GM 26543) and the National Science Foundation (Grant 8012722 CHE).

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system. After an additional 5 days, reduction of (±)-2-norbornanone was complete: the products were 72% *endo*-norbornanol and 28% *exo*-norbornanol. The enantiomeric purities of the α-methoxy-α-(trifluoromethyl)phenylacetate (MTPA) derivatives of *endo*- and *exo*-norbornanols were 38% and 100%, respectively. The residual activities of HLAD and D- and L-LDH after one experiment (8 days total) were 60%, 70%, and 63%, respectively, of the original immobilized activities. The residual aggregate activity of NAD^+ and NADH was 35% of the original value. The turnover numbers (TN = mol product/mol catalyst or cofactor) were as follows: $TN_{NAD} = 49$; $TN_{L_2Rh} = 1470$; $TN_{L\text{-}LDH} = 1.2 \times 10^7$, $TN_{D\text{-}LDH} = 2.5 \times 10^6$; $TN_{HLAD} = 7.5 \times 10^4$; $TN_{lactate} = 3$.

Reduction of Cyclohexanone. An analogous procedure was applied to the reduction of cyclohexanone. After 3 days, GLC analysis indicated 50% reduction; no further reaction occurred. Additional rhodium complex was added, and after 5 days (8 days total) cyclohexanol was obtained in 100% yield. The residual activities of the enzymes were 68% (D-LDH), 62% (L-LDH), and 59% (HLAD). The aggregate remaining activity of the nicotinamide cofactors was 38% of the original value. The turnover numbers were $TN_{NAD} = 50$, $TN_{L_2Rh} = 1500$, $TN_{L\text{-}LDH} = 1.2 \times 10^7$, $TN_{D\text{-}LDH} = 2.5 \times 10^6$, $TN_{HLAD} = 7.7 \times 10^4$, $TN_{lactate} = 3$.

Discussion

The systems described here require high starting concentrations of lactate and NAD^+ because of the highly unfavorable equilibrium (eq 1) for the conversion of lactate to pyruvate[9] and because

$$K = \frac{[\text{pyruvate}][\text{NADH}][H^+]}{[\text{lactate}][NAD^+]} = 2.76 \times 10^{-12}\ \text{M} \quad (1)$$

of the fact that the stability of the system is improved if these concentrations are greater than K_m. The Michaelis constants for NAD^+ and L-lactate for L-LDH are 0.25 mM and 6.7 mM, respectively;[10] the K_m values for NAD^+ and D-lactate for D-LDH are 1.2 mM and 70 mM, respectively.[11]

Control experiments established that ketone substrates of HLAD were not reduced, within our limits of detection, by complexes **1a** and **1b** after 8 days under experimental conditions. We chose 2-norbornanone as substrate in order to compare the product distributions with those obtained previously by Jones and co-workers using a conventional system.[12] Jones observed that the 2-norbornanol obtained under his conditions was >99% endo. We observed both *endo*- and *exo*-norbornanol by comparison of ^1H NMR spectra of the MTPA derivatives of the norbornanols formed with those of authentic samples. In agreement with Jones, we observed poor enantioselectivity when racemates (such as (±)-2-norbornanone) were reduced under V_{max} (high substrate, low enzyme) conditions.

The reactions which caused the gradual loss in activity of NAD^+ and NADH in solution have not been rigorously identified in these systems. Pig heart L-LDH by itself catalyzes conversion of NAD^+ to an inhibitory complex with pyruvate.[13] Acid-catalyzed hydration of NADH[14] or reaction of hydroxide ion with NAD^+[15] may also contribute to the loss of activity of the nicotinamide cofactors. In addition, **1a** and **1b** slowly catalyzed the reduction of NAD^+ to enzymatically active NADH, and probably also to the inactive 1,2- and 1,6-dihydropyridine isomers.

Initial high concentrations of rhodium complex are not recommended because of competitive bimolecular reactions that lead to precipitation of the rhodium complexes. These complexes also deactivate at low concentrations of substrate.

Conclusions

This paper has outlined the design of a catalytic system incorporating coupled organometallic and enzymatic components. This approach provides an interesting and direct method of coupling oxidation of H_2 to reduction of NAD^+ without involving hydrogenase.[3] The successful operation of the catalytic system depends on several characteristics of its components. First, the bis(phosphine)rhodium complexes **1a** and **1b** reduce pyruvate to lactate separately in the presence of NAD^+, NADH, and norbornanone. Second, D-LDH and L-LDH are very selective catalysts for the reduction of NAD^+ by lactate. Third, both D- and L-LDH are readily available. The racemic lactate produced in the rhodium-catalyzed reduction of pyruvate can thus be used efficiently. At present this system is less useful as a method of recycling NAD(H) than regeneration systems based on hydrogenase,[3] glucose 6-phosphate/glucose-6-phosphate dehydrogenase,[4] or formate/formate dehydrogenase. It has three practical disadvantages. First, the rhodium catalysts have only modest activity toward pyruvate ($TN \simeq 10\ h^{-1}$ under the conditions used here).[16] Second, the lifetime of the rhodium catalyst is limited. The deactivation of this component of the system ultimately limits the productivity and activity of this catalytic system. We have not identified the reactions leading to deactivation of the rhodium catalysts, but we note that these materials react with thiols and yield catalytically inactive products. The enzymes HLAD and D(L)-LDH contain thiols.[17] Third, the system (as most systems containing four cooperating catalytic activities) is not entirely straightforward to operate. In particular, efforts to carry out these reactions on much larger scales have not been reproducible. The origin of this failure in scale-up is almost certainly deactivation of the rhodium complex, but the course of the responsible processes has not been established.

Experimental Section

General. UV absorptions were monitored at 25 °C with a Perkin-Elmer 552 spectrophotometer. IR spectra were obtained on a Perkin-Elmer 598 spectrometer. $\{^1H\}^{31}P$ NMR spectra were recorded at 36.4 MHz on a modified Bruker HX-90 spectrometer. ^1H and ^{19}F NMR spectra were recorded at 250 and 84.26 MHz for determination of enantiomeric excesses on Bruker FX-90Q and Jeol WM-250 90 spectrometers, respectively. Gas chromatography was performed on a Perkin-Elmer 3920 B instrument equipped with a flame ionization detector, using a 10-ft 10% Carbowax column.

Materials. Cyclohexanone and (±)-2-norbornanone were obtained from Aldrich. (Norbornadiene)rhodium chloride was obtained from Strem. Enzymes and NAD were obtained from Sigma. Dioxane was distilled from sodium borohydride under argon. Water was deionized and distilled with a Corning Model 3B still. Hydrogen (prepurified grade) was passed through commercially available oxygen scavenger catalysts (Oxisorb).

Enzymes. All enzymes used here, whether obtained as suspensions in ammonium sulfate solutions or as lyophilized protein, were dialyzed twice against 150 mL of 50 mM Hepes buffer (pH 7.6) at 4 °C and concentrated by using an immersible-CX molecular separator kit (Millipore Corp.) before immobilization. Enzymes were immobilized by following general procedures described elsewhere,[18] except that DTT and other thiol-containing components were omitted.[19]

Assays. Assays of enzymes in homogeneous solutions were performed spectrophotometrically following the standard assay procedures described

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in Bergmeyer.[20] Activities were expressed in units of μmol min^{-1}.

Coimmobilization of Horse Liver Alcohol Dehydrogenase (HLADH), L-Lactate Dehydrogenase (L-LDH), and D-Lactate Dehydrogenase (D-LDH). Dialyzed HLAD (EC 1.1.1, 215 U), L-LDH (EC 1.1.1.27, 1395 U), and D-LDH (EC 1.1.1.28, 1714 U) were coimmobilized with PAN-1000.[18] Hepes buffer (24 mL, 0.3 M, pH 7.6) containing NAD^+ (3 mM), ethanol (850 mM), pyruvate (17 mM), and NADH (2 mM) was added to 6 g of PAN-1000 with stirring, followed by 4 mL of enzyme solution and TET (5 mL). Other details were the same as those of the general procedure described.[18] The immobilization yields were HLADH 58%, L-LDH 43%, and D-LDH 35%.

Bis(phosphine) Ligands for Complexes 1a and 1b. The water-soluble chelating diphosphine ligands used in the preparation of complexes **1a** and **1b** were synthesized following procedures described elsewhere.[21] Ligand **1a** was obtained in 100% yield: IR (neat) 3500–2500, 1710, 1630 cm^{-1}; ^{31}P NMR (H_2O) −19.1, −21.1 ppm. Ligand **1b** was obtained in 89% yield: IR (CH_2Cl_2) 1712, 1625, 1595, 1200, 1050 cm^{-1}; ^{31}P NMR (H_2O) −21.4, −23.1 ppm.

Bis(phosphine)rhodium Complexes 1a and 1b. Preparation of the cationic rhodium complexes **1a** and **1b** followed essentially the procedure described.[16] $[Rh(NBD)Cl]_2$ (3.6 mg, 8.0 μmol) and silver triflate (4.0 mg, 16 μmol) were placed in a 5-mL round-bottomed flask containing a small stirring bar. The flask was capped with a rubber septum and flushed thoroughly with argon. Dioxane (0.5 mL) was introduced and the mixture stirred for 5 min. The resulting yellow-orange solution was decanted from the AgCl precipitate by cannula into a solution of the appropriate chelating diphosphine ligand (19.2 mmol, 20% excess) in 1.0 mL of aqueous dioxane (1:1) and stirred for 15 min. Bright yellow homogeneous solutions of either rhodium complex **1a** or **1b**, as $L_2Rh(I)(norbornadiene)^+CF_3SO_3^-$, were obtained and were used without characterization.

Asymmetric Reduction of (±)-2-Norbornanone. A representative reaction was carried out at ambient temperature in a 300-mL pressure vessel containing a magnetic stirring bar. Sodium lactate (racemic, 1.8 g, 20 mmol), NAD^+ (0.86 g, 1.2 mmol), and (±)-2-norbornanone (6.6 g, 60 mmol) were dissolved in 50 mL of water, and the solution was adjusted to pH 8.0 with NaOH. Horse liver alcohol dehydrogenase (HLAD, 125 U), L-lactate dehydrogenase (L-LDH, 600 U), and D-lactate dehydrogenase (D-LDH, 600 U), coimmobilized in cross-linked polyacrylamide gel particles (PAN-1000, 150 mL of gel), were added as a suspension in 50 mL of water (pH 8.0). The pressure bottle was sealed and the reaction mixture flushed for 30 min with argon.[22] An aqueous dioxane solution of one of the rhodium complexes, **1a** or **1b** (0.016 mmol), was transferred by cannula into the reaction bottle. The system was purged with hydrogen for 5 min, the hydrogen pressure adjusted to 40 psi, and the reaction followed by GLC for norbornane derivatives and by enzymatic assays for NAD(H), pyruvate, and lactate. After 3 days, GLC analysis showed approximately 50% reduction, and no further reaction was observed. The reaction was continued by introducing an additional 0.024 mmol of rhodium complex into the system. After an additional 5 days the reaction was complete. The gel was allowed to settle, and the supernatant was decanted under positive pressure and continuously extracted with dichloromethane for 3 h. The CH_2Cl_2 extract was dried ($MgSO_4$) and concentrated to give a waxy white solid (6.6 g, 58.8 mmol) containing (by GLC) 72% *endo*-norbornanol and 28% *exo*-norbornanol. The mixture of norbornanols was converted to MTPA esters by use of freshly prepared (+)-(αR)-α-methoxy-α-(trifluoromethyl)phenylacetyl chloride.[23] The enantiomeric composition was determined by examination of ^{19}F NMR chemical shift differences for the α-CF_3 groups ($CDCl_3$, trifluoroacetic acid external standard): The α-CF_3 group absorption for the (+)-(1*S*,2*R*,4*R*)-endo diastereomer appeared at δ 7.158, and the absorption for the (−)-(1*R*,2*S*,4*S*)-endo diastereomer appeared at δ 7.071. From integration of these signals a 38% ee of the (+)-(1*S*,2*R*,4*R*)-*endo*-2-norbornanol was calculated. The α-CF_3 group absorption for (−)-(1*R*,2*R*,4*S*)-*exo*-2-norboranol appeared at δ 7.014: ee = 100%. The absolute configurations of the diastereomers were assigned on the basis of comparison with enantiomerically enriched reference samples prepared following standard resolution procedures.[24]

Hydrogenation of Pyruvate and NAD^+ by Complexes 1a and 1b. A solution of the bis(phosphine)rhodium complex **1a** or **1b** (16 μmol) prepared as described above was introduced into an argon-flushed reaction flask containing NAD^+ (0.86 g, 1.2 mmol) and pyruvate (1.0 g, 9.0 mmol) dissolved in 250 mL of water (pH 8.0). The system was purged with hydrogen for 5 min, the hydrogen adjusted to 40 psi, and the reaction followed by enzymatic assay for NADH and lactate. The following initial rates of hydrogenation were obtained: 8.8×10^{-8} mol/s (**1a**, pyruvate); 2×10^{-9} mol/s (**1a**, NAD^+); 6.7×10^{-8} mol/s (**1b**, pyruvate); 1×10^{-9} mol/s (**1b**, NAD^+).

Registry No. 1a, 71120-42-8; **1b**, 79971-91-8; L-LDH, 9001-60-9; D-LDH, 9028-36-8; NAD, 53-84-9; HLADH, 9031-72-5; (±)-2-norbornanone, 22270-13-9; cyclohexanone, 108-94-1.

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