

Enzyme-Catalyzed Transhydrogenation between Nicotinamide Cofactors and Its Application in Organic Synthesis¹

Chi-Huey Wong and George M. Whitesides*

Department of Chemistry
Massachusetts Institute of Technology
Cambridge, Massachusetts 02139

Received November 24, 1981

Several of the most convenient systems that have been proposed for the regeneration of the nicotinamide cofactors are specific for NAD(H).^{2,3} Although NADP(H) is less common in synthetically interesting biochemical pathways than NAD(H), on those occasions when NADP(H) is required, one is restricted for its regeneration to two procedures.^{4,5} Here we describe three practical systems that accomplish transhydrogenation between NAD(H) and NADP(H) cofactors (eq 1) and demonstrate their



application to problems in practical synthesis (Scheme I).⁶ These systems permit regeneration procedures that are specific for one cofactor (NAD(H) or NADP(H)) to be coupled to the other.

The first of these schemes utilizes the flavoenzyme diaphorase (EC 1.6.4.3) from *Clostridium klyveri*; the second uses alcohol dehydrogenase from *Leuconostoc mesenteroides* (ADH, EC 1.1.1.1) in a system containing both ethanol and acetaldehyde; the third involves glutamic dehydrogenase (GluDH, EC 1.4.1.3) in solutions containing glutamate (Glu), 2-ketoglutarate (2-KG), and ammonium ion. Added redox-active substrates are required

for transhydrogenation activity with ADH and GluDH but not for diaphorase,⁷ which contains FAD and which catalyzes hydride transfer between oxidized and reduced nicotinamide coenzymes directly. We used concentrations of the added substrates for ADH and GluDH that were 5–10*K*_m. Relevant kinetic parameters for these enzymes are summarized in Table I.

Demonstration of the coupling of a NADP⁺-specific enzymatic oxidation to a regeneration system specific for NADH → NAD⁺ was accomplished by conversion of pyruvate to L-lactate (**1**) and

(1) Supported by the National Institutes of Health (GM 26543 and GM 30367).

(2) Shaked, Z.; Whitesides, G. M. *J. Am. Chem. Soc.* **1980**, *102*, 7104.

(3) Jones, J. B.; Beck, J. F. In "Application of Biochemical Systems in Organic Chemistry"; Jones, J. B., Perlman, D., Sih, C. J., Eds.; Wiley-Interscience: New York, 1976; p 107–401.

(4) Wong, C.-H.; Whitesides, G. M. *J. Am. Chem. Soc.* **1981**, *103*, 4890–4899.

(5) Wong, C.-H.; Whitesides, G. M. *J. Org. Chem.*, in press.

(6) The flavoenzyme pyridine nucleotide transhydrogenase (EC 1.6.1.1) from *Pseudomonas* or spinach catalyzes direct transhydrogenation (Kaplan, N. O.; Colowick, S. P.; Neufeld, E. F. *J. Biol. Chem.* **1953**, *205*, 1–15). Since NADP⁺ is a potent dead-end inhibitor for this enzyme, the forward reaction (NADP⁺ + NADH → NAD⁺ + NADPH) is very slow (Cohen, P. T.; Kaplan, N. O. *J. Biol. Chem.* **1970**, *245*, 4666–4672). Moreover, the enzyme isolated from either source (ca. 20 units from 1 kg of spinach or *Pseudomonas* cells) has low specific activity (0.1–0.3 unit mg⁻¹). We did not attempt to use this enzyme for large-scale synthesis.

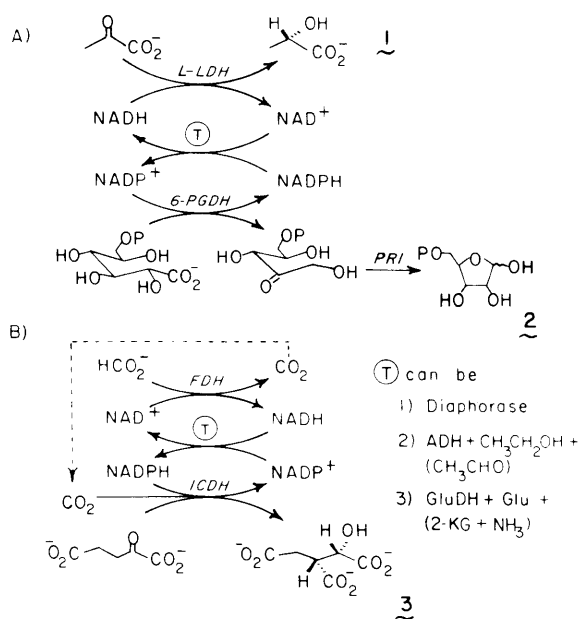
(7) Pig heart lipoamide dehydrogenase is essentially specific for NAD⁺ (NADP⁺ is only 5% as active a substrate as NAD⁺, in either the presence or the absence of added lipoic acid). Addition of lipoic acid to the transhydrogenation system containing diaphorase from *C. klyveri* did not improve its catalytic activity, because lipoic acid (or lipoamide) is a poor substrate for this enzyme.

Table I. Kinetic Parameters for Diaphorase, ADH, and GluDH

enzyme	K_m , mM					specific activity, ^a units mg ⁻¹ NADPH + NAD ⇌ NADP + NAD	
	NAD	NADH	NADP	NADPH	2nd substrate	for- ward	re- verse
diaphorase ^b (<i>C. kluyveri</i>)		0.1		0.1		2	3
ADH ^c (<i>L. mesenteroides</i>)	0.5	0.25	0.085	0.2	EtOH, 50 (NAD ⁺) 17 (NADP ⁺) CH ₃ CHO, 0.25 (NADH) 0.20 (NADPH)	7	10
GluDH ^d	0.02	0.03	0.047	0.026	Glu, 1.8 (NADP ⁺) 2-KG, 0.7 (NADP ⁺) NH ₄ ⁺ , 3.2 (NADPH)	2	2

^a The reaction mixture was quenched by adding an equal volume of 1 N NHClO₄ to destroy NAD(P)H and enzymes. The concentration of NADP was then determined by G-6-P/G-6-PDH, and NAD was determined by ethanol and ADH. ^b Wren, A.; Massey, V. *Biochim. Biophys. Acta* 1966, 122, 436-449. ^c Hatanaka, A.; Adachi, O.; Chiyonobu, T.; Ameyama, M. *Agr. Biol. Chem.* 1971, 35, 1304-1306. ^d Fahien, L. A.; Wiggert, B. O.; Cohen, P. P. *J. Biol. Chem.* 1965, 240, 1083-1090.

Scheme I. Regeneration of Nicotinamide Cofactors for Use in Enzyme-Catalyzed Synthesis Using Enzyme-Catalyzed Transhydrogenation: (A) Regeneration of NADH and NADP; (B) Regeneration of NAD and NADPH^a



^a Abbreviations: L-LDH, L-lactate dehydrogenase; 6-PGDH, 6-phosphogluconic dehydrogenase; PRI, phosphoriboisomerase; ADH, alcohol dehydrogenase; GluDH, glutamate dehydrogenase; ICDH, isocitrate dehydrogenase; FDH, formate dehydrogenase; Glu, glutamate; 2-KG, 2-ketoglutarate. The components in parentheses were not added but generated in the reaction.

6-phosphogluconic acid to ribose-5-phosphate (**2**). A 600-mL solution containing 6-phosphogluconate (0.2 M), pyruvate (0.2 M), NAD⁺ (0.2 mM), NADP⁺ (0.2 mM), mercaptoethanol (4 mM), and 200 units each of immobilized phosphoriboisomerase (PRI, in 6 mL of PAN⁸ gel), 6-phosphogluconate dehydrogenase (6-PGDH, in 8 mL of gel), diaphorase (in 20 mL of gel), and L-lactate dehydrogenase (L-LDH, in 0.5 mL of gel) was deoxygenated with Ar and stirred at 25 °C with the pH automatically controlled in the range 8.0-8.2 by adding 1 N KOH through a peristaltic pump. The reaction was complete in 8 days, and **1** and **2** were isolated as described previously:^{4,9} zinc lactate (15.3 g, 94% pure calculated as Zn(L-lactate)₂·2H₂O, 81% yield, 94% enantiomeric excess); barium ribose-5-phosphate (39.5 g, 92% purity determined enzymatically, 83% yield). The turnover

numbers and residual activities for each component at the conclusion of the reaction were as follows: NAD(H), 1000 (82%); NADP(H), 1000 (70%); 6-PGDH, 2 × 10⁶ (79%); PRI, 3 × 10⁶ (86%); diaphorase, 6 × 10⁵ (82%); L-LDH, 6 × 10⁷ (88%).

Replacement of diaphorase with ethanol (1.2% v/v) and immobilized ADH (from *L. mesenteroides*, 200 units in 5 mL of PAN gel) or glutamic acid (18 mM) and GluDH (200 units in 5 mL of PAN gel) resulted in very similar results with approximately the same TN for NAD(P)⁺.

The reverse (reductive) transhydrogenation was demonstrated by using the synthesis of *threo*-D₃(+)-isocitric acid (**3**). A 800-mL solution containing 2-KG (0.2 M), formate (0.2 M), NaHCO₃ (0.2 M), NAD⁺ (0.2 mM), NADP⁺ (0.2 mM), mercaptoethanol (4 mM), and 80 units each of immobilized isocitrate dehydrogenase (ICDH, in 20 mL of gel), diaphorase (in 10 mL of gel), and formate dehydrogenase (FDH, in 35 mL of gel) was deoxygenated and stirred at room temperature with pH controlled at 7.6. The reaction was 62% complete in 8 days, and no further reaction was observed.¹⁰ GluDH (100 units, in 3 mL of gel) and ammonium sulfate (60 mmol) were added to the reaction mixture to consume the unreacted 2-KG, and the reaction was allowed to proceed for 3 additional days. Compound **3** was isolated as its barium salt (82 g),⁴ which contained 88% (91 mmol) of Ba₃(isocitrate)₂ (57% yield). The turnover number and residual activity of each component was as follows: NAD(H), 620 (90%); NADP(H), 1000 (66%); ICDH, 1.6 × 10⁶ (88%); diaphorase, 4 × 10⁵ (78%); FDH, 1.5 × 10⁵ (80%).

A very similar result was obtained in this conversion of 2-KG to **3** when the diaphorase was replaced with ethanol (1.2% v/v) and ADH (200 units). The turnover number and residual activity of ADH was 8 × 10⁶ (80%); TN and residual activity for other components were similar to those observed in the system using diaphorase. The Glu/GluDH system could not be used in this instance, because the high concentration of 2-KG (present as a substrate for ICDH) inhibits the dehydrogenation of Glu.¹¹

This work establishes simple experimental protocols, using commercially available enzymes, that catalyze transhydrogenation between NAD(H) and NADP(H). Of the three reactions, that based on ADH has the highest rate and is the most convenient to use. It has, however, the disadvantage that the system must contain organic components (ethanol and acetaldehyde), which might deactivate some enzymes used in synthesis. The diaphorase-catalyzed transhydrogenation requires no added components but is kinetically slower (and more expensive) than that based on ADH.

(8) Pollak, A.; Blumenfeld, H.; Wax, M.; Baughn, R. L.; Whitesides, G. M. *J. Am. Chem. Soc.* 1980, 102, 6324-6336.

(9) Wong, C.-H.; McCurry, S. D.; Whitesides, G. M. *J. Am. Chem. Soc.* 1980, 102, 7938-7938.

(10) The extent of reaction was limited by the equilibrium constant of the ICDH-catalyzed reaction ($K = \frac{[2KG][CO_2][NADPH]}{[isocitrate][NADP^+]} = 0.77$ M; pH 7.0, 22 °C: Ochea, S. *Method. Enzymol.* 1955, 1, 699-704) and by product inhibition ($K_m(isocitrate) = 2.6$ M: Rose, Z. B. *J. Biol. Chem.* 1960, 235, 928-933).

(11) Fahien, L. A.; Wiggert, B. O.; Cohen, P. P. *J. Biol. Chem.* 1965, 240, 1083-1090.

These procedures for catalyzing transhydrogenation introduce an element of flexibility into nicotinamide cofactor regeneration and provide another method of controlling the redox behavior of systems of enzymes used for organic synthesis.

Registry No. NAD, 53-84-9; NADPH, 53-57-6; NADH, 58-68-4; NADP, 53-59-8; diaphorase, 37340-89-9; alcohol dehydrogenase, 9031-72-5; glutamic dehydrogenase, 9001-46-1; ribose-5-phosphate, 4300-28-1; L-lactate, 79-33-4; *threo*-D₃(+)-isocitrate, 6061-97-8; 6-phosphogluconic acid, 2464-13-3; pyruvate, 127-17-3; 2-ketoglutarate, 328-50-7.