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Efficient NAD^+-Regeneration System with Heterocyclic o-Quinones and Molecular Oxygen Catalyzed by Diaphorase

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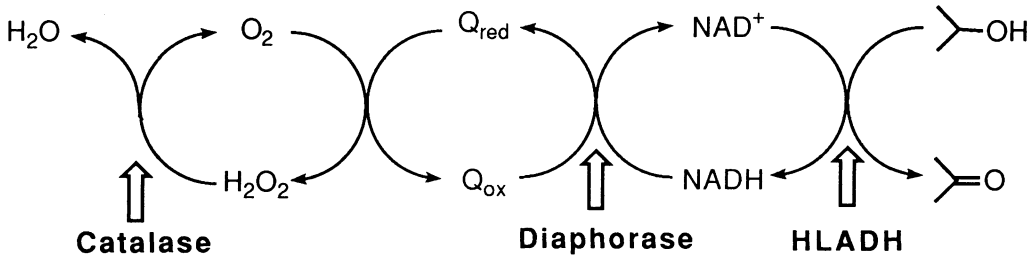
Addition of catalase and diaphorase drastically improved efficiency of the NAD^+-regeneration system with heterocyclic o-quinones and molecular oxygen.

Enzyme-catalyzed organic synthesis has recently attracted a great deal of attention upon its inherent high stereo-, substrate-, and chemoselectivities. In particular, NAD(P)(H)-requiring oxidoreductases are the most useful and widely investigated enzymes.[1] In order to use such enzymes for organic synthesis, development of an efficient regeneration system of the coenzyme is indispensable. Thus much effort has been made by using chemical, electrochemical, and enzymatic methods.[2] As a chemical method, we have demonstrated an NAD^+-regeneration system using heterocyclic o-quinones and molecular oxygen, which is much more efficient than the well known method of FMN/O_2.[3] In this paper, we would like to demonstrate that addition of catalase and diaphorase into our system produces an excellent improvement of the catalytic efficiency (Scheme 1).



Scheme 1.

At first, effect of diaphorase on the reaction between NADH and coenzyme PQQ (4,5-dihydro-4,5-dioxo-1H-pyrrolo[2,3-f]quinoline-2,7,9-tricarboxylic acid)[4] was examined in a neutral aqueous solution (pH 6.7), since this reaction was found to be the rate-determining step in our system.[3] The rate of the aerobic oxidation of NADH catalyzed by PQQ was increased markedly by the addition of diaphorase as shown in Fig. 1. The similar rate enhancement was also observed in the case of 1,7-phenanthroline-5,6-dione (1,7-PD) and 1,10-

phenanthroline-5,6-dione (1,10-PD) which have been also shown as the good catalysts (Table 1). Interestingly, the rate enhancement was extremely large in the case of the hydrophobic o-quinone, phenanthrenequinone (PQ), which itself is a poor catalyst in the absence of diaphorase. Since FMN and methylene blue (MB) have been used as catalysts in the similar system,[5,6] the catalytic efficiencies of those catalysts were also examined under the same conditions. However, the rates were not so high even in the presence of diaphorase as compared with those of the *o*-quinones.

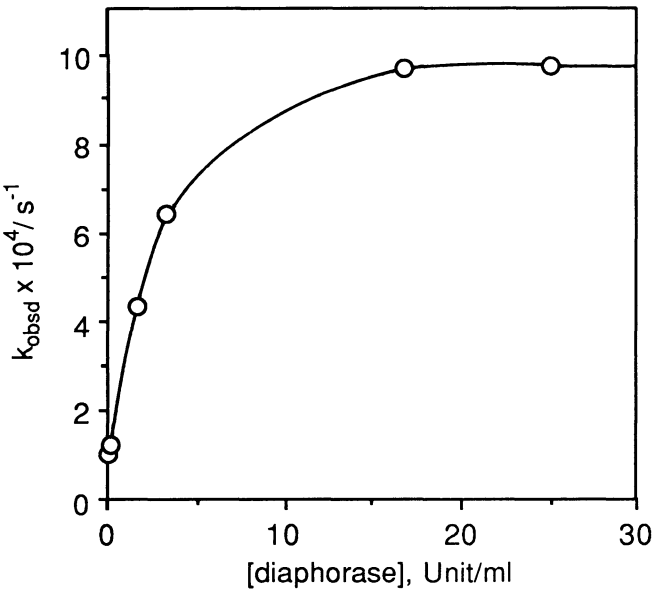


Fig. 1. Effect of diaphorase on the aerobic oxidation of NADH (4.0 x10^{-4}M) catalyzed by PQQ (4.0 x 10^{-6}M) in 0.1 M phosphate buffer (pH 6.7) at 30 °C.

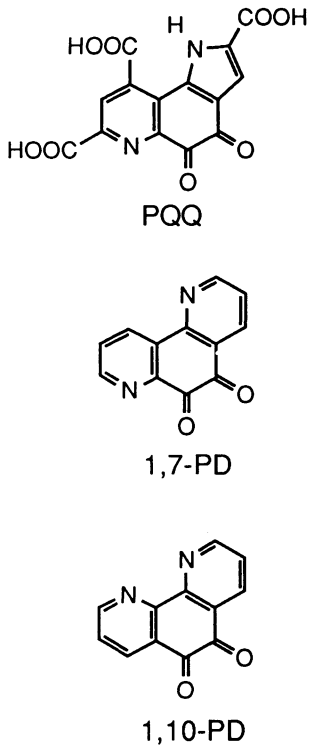


Table 1. First-order rate constant of the aerobic oxidation of NADH [a]

Catalyst	k_{obsd} x 10^4 / s^{-1}	
	without diaphorase	with diaphorase
PQQ	1.03	6.43
1,7-PD [b]	1.75	5.82
1,10-PD [b]	2.31	5.34
PQ [b]	0.36	37.9
MB	0.36	3.04
FMN	0.22	0.54
–	0.22 [c]	0.42

a) [catalyst] = 4.0 x 10^{-6}M, [NADH] = 4.0 x 10^{-4}M, [diaphorase] = 3.3 U/ml 0.1 M phosphate buffer (pH 6.7), under aerobic conditions, 30 °C. b) The solution contains 1% CH_3CN. c) The rate of hydration of NADH.

The aerobic autorecycling oxidation of NADH was then applied to the HLADH-catalyzed oxidation of alcohols (Scheme 1). In Fig. 2 is shown the time course of the oxidation of cyclohexanol (2.0 x 10^{-3}M) in the presence of a catalytic amount of HLADH (5 x 10^{-7}M), PQQ (1.0 x 10^{-4}M), and NAD^+ (1.0 x 10^{-4}M) in 0.1 M phosphate buffer (pH 8.2) at 30 °C under aerobic conditions. Addition of catalase (625 U/ml) into the reaction mixture improved the catalytic efficiency as mentioned before.[3] Catalase may prevent oxidative deactivation of the enzyme and the catalysts by hydrogen peroxide which is produced during the catalytic cycles. Furthermore, addition of diaphorase (8.6 U/ml) together with catalase drastically accelerated the reaction rate and cyclohexanol was oxidized to cyclohexanone almost quantitatively within 3 h.

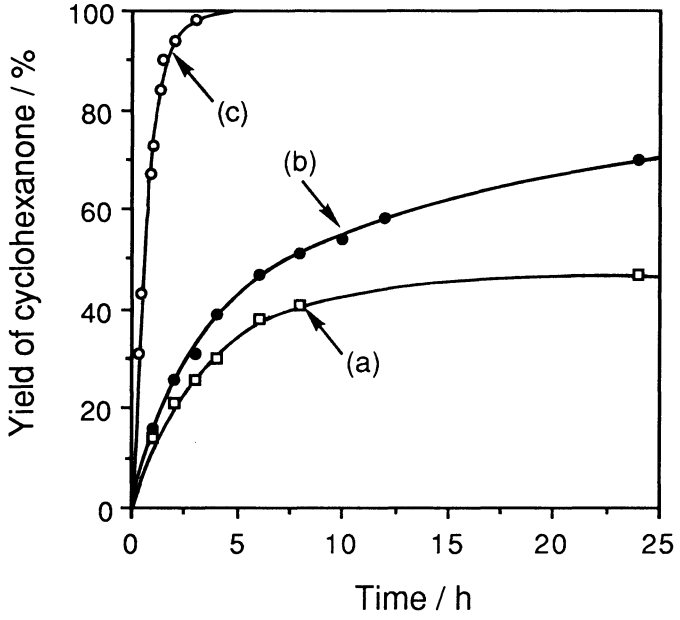


Fig. 2. Time course of the oxidation of cyclohexanol (2.0 x 10^{-3}M) by using the system of (a) HLADH (5.0 x 10^{-7}M) - NAD^+ (1.0 x 10^{-4}M) - PQQ (1.0 x 10^{-4}M), (b) HLADH (5.0 x 10^{-7}M) - NAD^+ (1.0 x 10^{-4}M) - PQQ (1.0 x 10^{-4}M) - catalase (625 unit/ml), and (c) HLADH (5.0 x 10^{-7}M) - NAD^+ (1.0 x 10^{-4}M) - PQQ (1.0 x 10^{-4}M) - catalase (625 unit/ml) - diaphorase (8.6 unit/ml) in O_2 saturated 0.1 M phosphate buffer (pH 8.2) at 30 °C.

The effects of catalase and diaphorase were also examined in the reactions with phenanthrolinequinone derivatives (1,7-PD and 1,10-PD), phenanthrenequinone (PQ), methylene blue (MB), and FMN (Table 2). Catalase improved the oxidation yields in all the cases except FMN, but enhancement of the reaction rates was not so satisfactory (it takes about 24 h). As in the case of PQQ, diaphorase drastically enhanced the catalytic efficiencies of the heterocyclic *o*-quinones, and the oxidation of cyclohexanol proceeded almost quantitatively within 4 h. However, such a high efficiency was not obtained in the case of FMN even in the presence of catalase and diaphorase. Although PQ and MB were not so

good catalysts in the absence of diaphorase, addition of the enzyme also improved the oxidation yields. These results may indicate that the reaction between NADH and the catalyst is no longer the rate limiting step under these conditions. Further studies including kinetic analysis are now in progress.

Table 2. Oxidation of cyclohexanol by the HLADH-NAD^+-catalyst-O_2 system [a)]

Catalyst	Yield / % of cyclohexanone		
	none	with catalase [b)]	with catalase and diaphorase [c)]
PQQ	47	75	99
1,7-PD [d)]	43	80	90
1,10-PD [d)]	44	83	95
PQ [d)]	11	63	94
MB	17	47	97
FMN	10	18	69

a) [catalyst] = [NAD^+] = 1.0 x 10^{-4}M, [cyclohexanol] = 2.0 x 10^{-3}M, [HLADH] = 5.0 x 10^{-7}M, 0.1 M phosphate buffer (pH 8.2), under dark and aerobic conditions, *reaction time = 24 h.* b) [catalase] = 625 U/ml, *reaction time = 24 h.* c) [catalase] = 625 U/ml, [diaphorase] = 8.6 U/ml, *reaction time = 4 h.* d) The solution contains 2.5% CH_3CN.

So far, FMN is usually used as an aerobic autorecycling catalyst for the regeneration of NAD^+ from NADH,[5,7] but the present results clearly indicate that the heterocyclic o-quinones are the best catalysts for practical application.

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