Notes

A Novel Water-Soluble Hantzsch 1,4-Dihydropyridine Compound That Functions in Biological Processes through NADH Regeneration

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Abstract: A novel Hantzsch 1,4-dihydropyridine derivative could function in an organic solvent-free solution, and thus it could function in biological systems. These functions can be accomplished through regeneration of the reduced form of nicotinamine adenine dinucleotide (NADH), an essential compound for living organisms. The results obtained here demonstrate the usefulness of a water-soluble Hantzsch 1,4-dihydropyridine derivative and its wide applicability as a chemical energy source, which drives various biological processes efficiently.

Food taken in by living organisms is ultimately processed into protons. By means of respiration that involves many oxidation−reduction reactions, the protons are pumped outside biological membranes to form an electrochemical proton gradient (proton motive force). Reentrance of the protons through the membranes then occurs, which couples with the synthesis of adenosine 5′-triphosphate (ATP), the universal biological energy currency. All living organisms, from humans to bacteria, would die without ATP, because it powers many important cellular processes.

We have been interested in whether organic compounds can regulate biological processes, aiming at application setups. We found that a novel Hantzsch 1,4-dihydropyridine derivative (the potassium salt of 2,6-dimethyl-1,4-dihydropyridine-3,5-dicarboxylic acid monomethyl ester,[1] herein designated as DPH; Figure 1) could reduce nicotinamine adenine dinucleotide (NAD^+), methylene blue, and cytochrome *c* in a nearly stoichiometric manner at pH 7.5 without an organic solvent. In contrast, DPH could not reduce flavin adenine dinucleotide or flavin mononucleotide, even in an excess amount. These findings suggest that DPH can reduce biological materials with some specificity under physiological conditions; thus, it possibly enhances many biological processes.

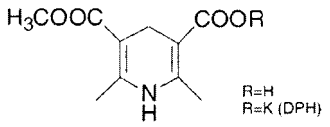

Figure 1. Structure of DPH.

In this note we report the functions of DPH in an enzymatic reaction and also in energy conversion systems that reside in biological membranes. These functions can be accomplished through regeneration of the reduced form of nicotinamine adenine dinucleotide (NADH), an essential compound for living organisms, in which it acts as an electron and proton carrier in many oxidation−reduction reactions. Regeneration of NADH cofactor is important in the field of catalysis, where biological processes are incorporated into application setups.[2,3] The results obtained here demonstrate the usefulness of water-soluble DPH and its wide applicability as a chemical energy source, which drives various biological processes efficiently through NADH regeneration.

Regeneration of NADH from NAD^+ by DPH in buffered water (pH 7.5) was first analyzed by liquid chromatography with wavelength detection at 340 nm. Quantitative analysis showed that 90% of the NAD^+ was reduced to NADH within 5 min at 25 °C after equal amounts of NAD^+ and DPH had been mixed (results not shown). We then examined whether DPH could be functionally applicable to an enzymatic reaction requiring NADH as a cofactor, where the reaction must proceed in water without an organic solvent. Horse liver alcohol dehydrogenase[4] (hADH, EC 1.1.1.1) reduces cyclohexanone to produce cyclohexanol in the presence of a stoichiometric amount of NADH as the substrate. We assayed the hADH activity on cyclohexanone reduction under physiological conditions with or without DPH. With 5 mM NAD^+, 71% of 5 mM cyclohexanone was steadily reduced in the presence of 5 mM DPH (Table 1). When the maximum 90% reduction of NAD^+ by a stoichiometric amount of DPH (see above) was taken into account, the efficiency obtained in this enzymatic reaction was consistent with the substrate reduction with only the natural cofactor NADH (5 mM): i.e., 85% reduction. The two-step additions of DPH (5 mM each) in the presence of 5 mM NAD^+ resulted in 97% reduction of the substrate. We also confirmed that cyclohexanone reduction by hADH did not occur in the presence of NAD^+ or DPH alone. From these results together, we conclude that DPH can reduce NAD^+ to form NADH, which is then used as a cofactor for the hADH enzymatic function.

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(1) The novel Hantzsch 1,4-dihydropyridine compound DPH was prepared as described elsewhere. DPH could be dissolved in water to as high as 200 mM and showed absorption maxima at 230 and 360 nm (ϵ = 8000 and 4000, respectively). To the best of our knowledge, DPH is one of the most soluble compounds in water among the 1,4-dihydropyridine derivatives previously reported.[5]

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(4) Sund, H.; Theorell, H. *The Enzymes*; Academic Press: New York, 2nd ed.; **1963**, *7*, 25−83.

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Table 1. Effects of DPH on the Reduction of Cyclohexanone by HADH

concn (mM)			yield of cyclohexanol (%)[a]	$NAD^+ \rightarrow$ NADH (recycles)
DPH	NAD^+	NADH		
5	5		70.6 ± 7.1	0.7
5	1		63.1 ± 6.1	3.2
5	0.2		29.7 ± 1.8	7.4
5	0.05		10.0 ± 2.0	10.0
		5	84.8 ± 1.7	
5			0	
	5		0	
5		5	98.9 ± 2.2	
5 + 5[b]	5		96.5 ± 4.0	1.0

[a] The mean values of the yields for at least three independent assays are shown, along with the standard deviation. [b] Cyclohexanone reduction was first carried out with 5 mM DPH for 2 h, and subsequently another 5 mM DPH was added for a further 4 h incubation.

Using the same enzymatic reaction by hADH, we next examined how many times single NAD^+ molecules could be recycled to regenerate NADH with the addition of DPH. We fixed the concentrations of DPH and cyclohexanone (both 5 mM) and assayed hADH (1 μM) activity with various concentrations of NAD^+ (Table 1). We found that NAD^+ could be recycled up to 10 times with the lowest amount of NAD^+ used (0.05 mM). Limitation of the ability to recycle NAD^+ by DPH (10 times) may be caused by unknown deleterious conversion in the minor population, as shown by 90% regeneration of NADH (see above). This recycling value was comparable to those obtained by Taylor and Jones[5] with other dihydropyridine compounds. However, as we used a 3 times lower amount of the reagent (DPH) compared with that used in their case, the recycling efficiency per DPH molecule appeared to be 3-fold better than those previously reported.[5]

We further explored the possibility of the DPH function being applied to a biological system. An enteric bacterium, *Escherichia coli*, can form a proton motive force driven by a membrane protein, NADH dehydrogenase; thus, it accumulates protons inside inverted membrane vesicles in the presence of NADH.[6] Figure 2 shows the formation of a proton gradient by *E. coli* membrane vesicles. We first added 0.5 mM NAD^+ to the membranes, no proton gradient being observed (see legend to Figure 2 for experimental details). To this mixture, we then added 0.5 mM DPH after 2 min, observing a significant level of acridine orange quenching, an indication of proton gradient formation. The quenching level in the presence of 0.5 mM NAD^+ and 0.5 mM DPH was similar to that with 0.5 mM NADH alone (Figure 2). A proton gradient was not formed with only 0.5 mM DPH. These results demonstrated that DPH could energize biological membranes through NADH regeneration from NAD^+ under physiological conditions.

The energized membranes can be used to synthesize ATP through a membrane-bound enzyme, ATP synthase, in vitro.[7] We also examined ATP synthesis via the DPH function using the energized membrane (Figure 3). In the presence of *E. coli* inverted membrane vesicles, DPH, NAD^+, ADP, and inorganic phosphate, we could obtain 7% conversion from ADP to ATP. Significantly, this value

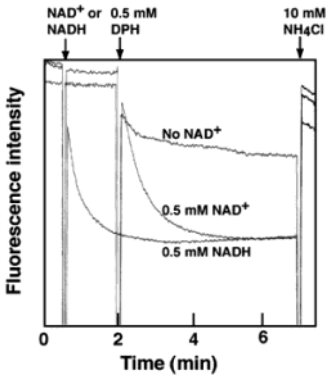

Figure 2. Effects of DPH on the formation of a proton gradient by *E. coli* membranes. At 30 s, 0.5 mM NAD^+ or NADH was added, and then at 2 min, DPH (0.5 mM) was further added. A decrease in the fluorescence intensity of acridine orange (quenching) represents the formation of a proton gradient, which disappears with the addition of 10 mM NH_4Cl at 7 min.

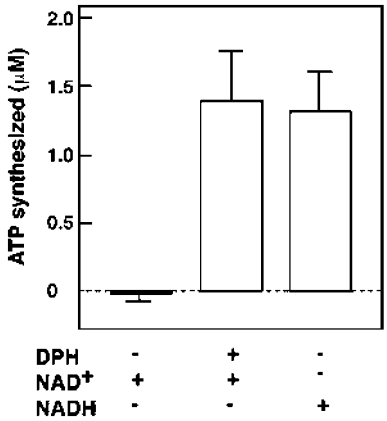

Figure 3. Effect of DPH on ATP synthesis. The mean values of the ATP contents for at least three independent assays are shown with the standard deviation.

was the same as in the case of NADH instead of DPH and NAD^+. ATP synthesis did not occur without DPH in the presence of NAD^+ (Figure 3). These results suggest that DPH is the trigger of the chemical energy (ATP) production in this system.

In this study, we showed that DPH could function in an organic solvent-free solution, and thus it can function in biological systems. DPH has a unique carboxyl moiety, which allows it to easily generate a potassium salt; thus, it is sufficiently soluble in water. This property takes advantage of the regeneration of the NADH cofactor. A large number of enzymes depend on this cofactor for their activities. However, the high cost of NADH is an important commercial issue, because a stoichiometric amount of the cofactor, as a substrate, is required in enzyme reactions. Cofactor regeneration must be, therefore, included in application setups when the systems involve NADH-dependent reactions. DPH can be incorporated into such systems.

Experimental Section

Cyclohexanone Reduction by hADH. All reactions were carried out for 4 h at 25 °C in aqueous 20 mM HEPES/NaOH buffer, pH 7.5, containing 5 mM cyclohexanone and 1 μM hADH. Further incubation did not cause an increase in the cyclohexanol yield. Consumption of cyclohexanone and production of cyclo-

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(6) Reenstra, W. W.; Patel, L.; Rottenberg, H.; Kaback, H. R. *Biochemistry* **1980**, *19*, 1−9.
(7) Etzold, C.; Deckers-Hebestreit, G.; Altendorf, K. *Eur. J. Biochem.* **1997**, *243*, 336−343.

hexanol were quantitatively assayed by gas chromatography after extraction with chloroform. Known concentrations of cyclohexanone and cyclohexanol were incubated in the same solution as used for the enzymatic reaction and then extracted with chloroform followed by gas chromatography to generate standard calibration curves. The mean values of the yields (percent) for at least three independent assays are shown with the standard deviation.

Proton Gradient Formation and ATP Synthesis. *E. coli* inverted membrane vesicles containing 90 μg protein were assayed for a proton gradient formation in 2 mL of 10 mM tricine/choline, pH 8.0, 140 mM KCl, 5 mM $MgCl_2$, and 1 μM acridine orange at 25 °C.[8] To the same reaction mixture as that used for assaying the proton gradient were further added 20 μM ADP and 1 mM KH_2PO_4, and then the effects of DPH, NAD^+, and NADH (each 0.5 mM) on ATP synthesis were examined. The reaction was terminated by the addition of 1 N trichloroacetic acid after 20 min of incubation at 25 °C. Further incubation and an increase in the DPH concentration (up to 1.5 mM) did not cause an increase in the amount of ATP synthesized. The ATP content was quantitatively determined with a bioluminescent assay kit (Sigma).

(8) Moriyama, Y.; Iwamoto, A.; Hanada, H.; Maeda, M.; Futai, M. *J. Biol. Chem*. **1991**, *266*, 22141−22146.

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