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Accelerated Publications

A Novel Minimum Ribozyme with Oxidoreduction Activity

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Received August 21, 1990; Revised Manuscript Received October 1, 1990

ABSTRACT: A nucleoside catalyzing the oxidoreduction of NADH and $K_3Fe(CN)_6$ was isolated from *Torula* yeast RNA and also obtained in 0.05% yield by a series of steps: SDS–phenol extraction, nuclease P_1 digestion, alkaline phosphatase digestion, anion exchange chromatography, and HPLC on an ODS column. Its chemical structure was clearly determined at 5-hydroxycytidine, from the results of FAB-MS and ^1H and ^{13}C NMR spectroscopies. The mass spectra, chromatographic behavior, UV spectra, and NMR spectra of this nucleoside from natural and synthetic sources were identical. This is the first report of an RNA catalyst having catalytic activity except for the cleavage and ligation of phosphodiester bonds of RNA. That an RNA has oxidoreduction activity indicates new possibilities for RNAs as "living molecules". 5-Hydroxycytidine may be a vestige of RNAs that formerly possessed metabolizing ability.

Until quite recently, RNA was considered a passive carrier of information stored in DNA (Crick, 1970). In the last few years, it has been found to have self-catalytic activity for splicing (Cech, 1981) and cleavage (Stark et al., 1978) of RNA, thus causing a change of image. Recent new results strongly support the possibility that RNA, not DNA, is the first carrier of information and is capable of replicating itself in the absence of protein enzymes (Gilbert, 1986; Sharp, 1985). Both these features may have been possessed by the same molecule in the early stages of biological evolution. Such RNAs are presently regarded as the legacy of primordial RNA (Pace & Marsh, 1985; Joyce, 1989).

Known catalytic RNAs (ribozymes) (Cech & Bass, 1986; Cech, 1987) can be categorized as group I RNA IVS[1] (Cech,

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[1] Abbreviations: IVS, intervening sequence; RNP, ribonucleoprotein; FAB-MS, fast atom bombardment mass spectrometry; HPLC, high-performance liquid chromatography; ODS, octadecylsilane; SDS, sodium dodecyl sulfate; TLC, thin-layer chromatography; h^5C, 5-hydroxycytidine; 2D NMR, two-dimensional nuclear magnetic resonance; k_{cat}, catalytic rate constant; K_m, Michaelis constant; 2,6-DCIP, 2,6-dichlorophenolindophenol; β-NMNH, β-nicotinamide mononucleotide, reduced form; β-NHypDH, β-nicotinamide hypoxanthine dinucleotide, reduced form; 3-AcPyADH, 3-acetylpyridine adenine dinucleotide, reduced form.

0006-2960/90/0429-10585$02.50/0

1988), group II RNA IVS (Michel et al., 1989), RNase P (Guerrier-Takada et al., 1983), and hammerheads (Symons, 1989) (which include several plant viral satellite RNAs, one viroid, and a transcript of a newt satellite DNA). However, these ribozymes (McSwiggen & Cech, 1989) are limited to sequence-specific cleavage and ligation of phosphodiester bonds of RNA. The chemical properties of various functional groups, such as ribose 2′-hydroxyl and nitrogen and keto groups in purine and pyrimidine rings within an RNA molecule, indicate possibly a broader range of catalytic function.

Coenzymes are complex organic molecules essential to many protein enzyme-catalyzed reactions. Many coenzymes are nucleotides (NAD^+, $NADP^+$, FAD, CoA, and ATP) or their related compounds (thiamin pyrophosphate, tetrahydrofolate, and pyridoxal phosphate). Coenzymes may be surviving vestiges of RNA (White, 1976) that existed prior to RNP and DNA.

Primordial RNAs may have possessed the ability to metabolize, such as catalyze reactions involving the transfer of electrons, hydrogen atoms, and acyl and alkyl groups (Hartman, 1975), and ribozymes were certainly present in the early stages of evolution. A search for ribozymes with oxidoreduction activity was made by the authors. In the following, the purification, structural analysis, and kinetics of an RNA component possessing oxidoreduction activity are discussed.

Materials and Methods

General Methods. *Torula* yeast RNA was purchased from Sigma Chemical Co., nuclease P_1 was obtained from YAMASA SHOYU Co., Ltd., *Escherichia coli* alkaline phosphatase was from TOYOBO Co., Ltd., and diaphorase (EC 1.8.1.4) was from *Clostridium kluyveri* from Oriental Yeast Co., Ltd. Ultraviolet spectra were measured on a Gilford RESPONSE II spectrophotometer. ^1H and ^{13}C NMR spectra were obtained with a Bruker AM 600-MHz spectrometer using Me_4Si as internal standard at 25–50 °C. FAB-MS was taken on a JEOL HX100 spectrometer by using diethanolamine as a matrix. HPLC was carried out on an IRICA 852 I liquid chromatograph.

Assay of Catalytic Activity. Oxidoreduction activity was assayed at 30 °C on the basis of the decrease in absorbance at 340 nm due to the oxidation of NADH. An assay mixture for oxidoreduction activity contained, at a final volume of 0.25 mL, 12.5 μmol of Tris-HCl buffer, pH 8.26, 0.025 μmol of $K_3Fe(NC)_6$, 0.05 μmol of NADH, and an appropriate amount of an RNA fraction. Reaction velocity was calculated after subtraction of the velocity of spontaneous reaction.

Purification of an RNA Component Catalyzing the Oxidoreduction of NADH and $K_3Fe(CN)_6$. Torula yeast (4 g) was suspended in 40 mL of water and completely dissolved by neutralization with 12.5 mL of 1 N NaOH. For deproteinization, the solution was treated with SDS at a final concentration of 0.5% for 1 h and then treated with 60 mL of phenol solution saturated with 0.1 M Tris-HCl (pH 8) at 25 °C. After being shaken for 20 min, the solution was centrifuged at 10000 rpm for 15 min. The pooled aqueous phase was reextracted with a mixture of 30 mL of phenol and 30 mL of chloroform/isoamyl alcohol (24:1) and centrifuged at 10000 rpm for 15 min. This phenol extraction procedure was repeated at least twice. The upper aqueous phase was extracted with 60 mL of a 24:1 mixture of chloroform and isoamyl alcohol to remove any lingering traces of phenol from the RNA preparation and centrifuged at 10000 rpm for 15 min. Sodium chloride was added to the supernatant fluid to bring the final concentration to 0.182 M, and then 2.6 volumes of ice-cold ethanol was added to the solution, mixed well, and stored at −20 °C overnight. The precipitate was collected by centrifugation at 10000 rpm for 15 min. The collected precipitate was then washed with two 120-mL portions of cold 70% ethanol and dried in vacuo. This ethanol precipitation was conducted at least twice.

The purified RNA was hydrolyzed to nucleosides with nuclease P_1 and alkaline phosphatase. The purified RNA (3.4 g) was dissolved in 160 mL of 0.05 M acetate buffer, pH 5.3, and digested with 1000 units of nuclease P_1 at 37 °C. After 18 h, the digestion was terminated by the addition of 15 mL of 1.5 M Tris-HCl buffer, pH 8. The nuclease P_1 treated RNA lysate was then treated with 260 units of alkaline phosphatase in the presence of 1.1 mM $MgCl_2$ at 37 °C for 18 h.

The nucleoside mixture was fractionated by anion-exchange chromatography on an AG 1-X2 (200–400 mesh, formate form, Bio-Rad Laboratories) column (3 × 25 cm). The alkaline phosphatase treated RNA lysate was diluted with water to 2.8 L, applied on an anion exchange column, washed with 3 L of water, and eluted with 0.02 M formic acid (for column chromatography, Nacalai Tesque, Inc.). The catalytic activity of the eluate from the chromatogram was monitored by NADH oxidation coupled with $K_3Fe(CN)_6$. The fraction with oxidoreduction activity was eluted with 0.02 M formic acid, collected, and lyophilized. This fraction (12 mg) was subjected to HPLC using an ODS column (MCI-GEL ODS 2PU, 1 × 30 cm, Mitsubishi Chemical Industries Ltd.). The elution solvent was 1% methanol (for HPLC, Wako Pure Chemical Industries, Ltd.)/0.02 M ammonium formate (specially prepared reagent, Nacalai Tesque, Inc.), pH 4. The flow rate was 1 mL/min, and the column effluent was monitored at 260 nm. The nucleoside fraction with oxidoreduction activity was eluted at 37 min. A pure nucleoside (2 mg) was obtained after lyophilization.

Results and Discussion

Purification of h^5C. The oxidoreduction activity of RNAs from different sources such as bakers' yeast, *Torula* yeast, calf liver, and *E. coli* was assessed. *Torula* yeast RNA was found to express the highest activity of all RNAs examined. Enzymatic digests of *Torula* yeast RNA exhibited oxidoreduction activity, and thus an attempt was made to isolate RNA components with oxidoreduction activity from enzymatic digests of *Torula* yeast RNA.

Torula yeast RNA was hydrolyzed to nucleosides with nuclease P_1 and alkaline phosphatase. The nucleoside mixture was fractionated by anion exchange chromatography on an AG 1 column, using 0.02 M formic acid for the elution. Cytidine, uridine, adenosine, and most of the guanosine were eluted together by washing the column with distilled water. However, no active component causing oxidoreduction of NADH and $K_3Fe(CN)_6$ was eluted with distilled water. The active component thus possibly has one negative charge at neutral pH. A small OD_{260} peak (fraction 1) emerging with the solvent (0.02 M formic acid) front was quite active for oxidoreduction activity. About 60% of the total oxidoreduction activity was recovered in fraction 1 while the rest appeared in three peaks that eluted later. However, the activity of any of these failed to exceed that of fraction 1. Cytidine, uridine, adenosine, and guanosine showed no oxidoreduction activity.

Fraction 1 was further fractionated by HPLC on an ODS column. The component possessing oxidoreduction activity was eluted as a single peak at 37 min. Its TLC on a cellulose plate (solvent: isopropyl alcohol/concentrated ammonia/water = 7:1:2) indicated a single ultraviolet-absorbing spot (R_f 0.23).

Table I: Purification of an RNA Component Catalyzing the Oxidoreduction of NADH and $K_3Fe(CN)_6$

purification step	total RNA mg	total RNA A_{260} units	total activity (nmol/min)	sp activity (nmol min^{-1} mg^{-1})	purification yield (%)	purification x-fold
(1) RNA from yeast (*Torula utilis*)	4000	109 720	2880	0.72	100	1
(2) SDS–phenol extraction	3400	64 838	1734	0.51	60	0.7
(3) nuclease P_1 digestion	3400	89 568	2584	0.76	90	1.1
(4) alkaline phosphatase digestion	3400	75 208	1870	0.55	65	0.8
(5) chromatography on an anion exchange column	12	264	4572[a]	381	150	530
(6) HPLC on ODS	2	27	6920	3460	240	4800

[a] Anomalous excess total activity in this step is probably due to removal of some inhibitors.

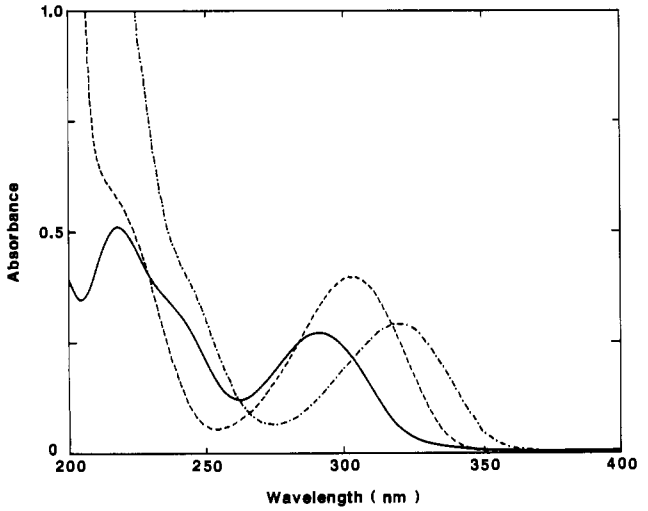


FIGURE 1: UV spectra of 5-hydroxycytidine: (—) in water; (- - -) in 0.1 M HCl; (···) in 0.1 M NaOH.

A portion of a single peak on the ODS column was pooled and used to determine the structure of the active component (**1**). The results obtained after each step of the preparation leading to the isolation of this component are summarized in Table I. Two milligrams of **1** was obtained as the pure active component from 4 g of Torula yeast RNA.

The most important information for the structure of **1** was obtained by FAB-MS. A negative ion peak (M − 1) appeared at 258, thus indicating its molecular weight to be 259.

About 1 mg of pure **1** obtained as above was subjected to NMR measurements. The presence of an unmodified ribose was deduced from the resonance at 5.89 ppm (1 H, d, 1′H, $J_{1'-2'}$ = 4.4 Hz), 4.27 ppm (1 H, t, 2′H, $J_{2'-3'}$ = 5.1), 4.20 ppm (1 H, t, 3′H, $J_{3'-4'}$ = 5.5 Hz), 4.11 ppm (1 H, q, 4′H, $J_{4'-5'}$ = 3.2, 4.6 Hz), and 3.80 and 3.91 ppm (2H, A-B pattern, 5′H, $J_{5'}$ = −12.7 Hz). Relative chemical shifts and coupling constants were typical of those for pyrimidine nucleosides and nucleotides (Uhl et al., 1983). The assignments of 1′H, 2′H, 3′H, 4′H, and 5′H were confirmed by ^1H 2D NMR spectroscopy. There were two resonances not assignable to the ribose moiety in the region downfield from the residual proton peak of water: a singlet signal at 7.44 ppm and a singlet at 8.45 ppm. A proton at 8.45 ppm was assigned to the formyl proton of formic acid. In the final purification step of **1**, a mixture of methanol and ammonium formate was used as the elution solvent of HPLC. **1** appeared to be present as a formic acid salt. A proton at 7.44 ppm was exchangeable; most of the proton disappeared on standing in D_2O at 50 °C for 3 days. This proton may thus possibly be an enolic proton. It was attributed to H6 of the pyrimidine ring moiety of **1**. It is thus evident that a hydroxyl group is attached to C5 of the pyrimidine ring moiety.

The presence of a hydroxyl group was also supported by the elution profile on an anion exchange column and UV absorption spectral data. **1** appeared to possess one negative charge, since it eluted after nucleosides from an anion exchange column. As shown in Figure 1, the UV spectrum of **1** showed a maximum at 292 nm (ϵ 7.19 × 10^3) in water, 303.5 nm (ϵ 10.4 × 10^3) in 0.1 M HCl, and 319 nm (ϵ 7.64 × 10^3) in 0.1 M NaOH, respectively. Major shifts of the spectra in acidic and alkaline solutions indicated the possible presence of amino and hydroxyl groups in the pyrimidine ring.

The ^{13}C NMR spectrum of **1** showed resonances from nine carbons. An unmodified ribose was considered present from the resonances at 92.72 ppm (1′C), 86.59 ppm (4′C), 76.50 ppm (2′C), 72.18 ppm (3′C), and 63.62 ppm (5′C). There

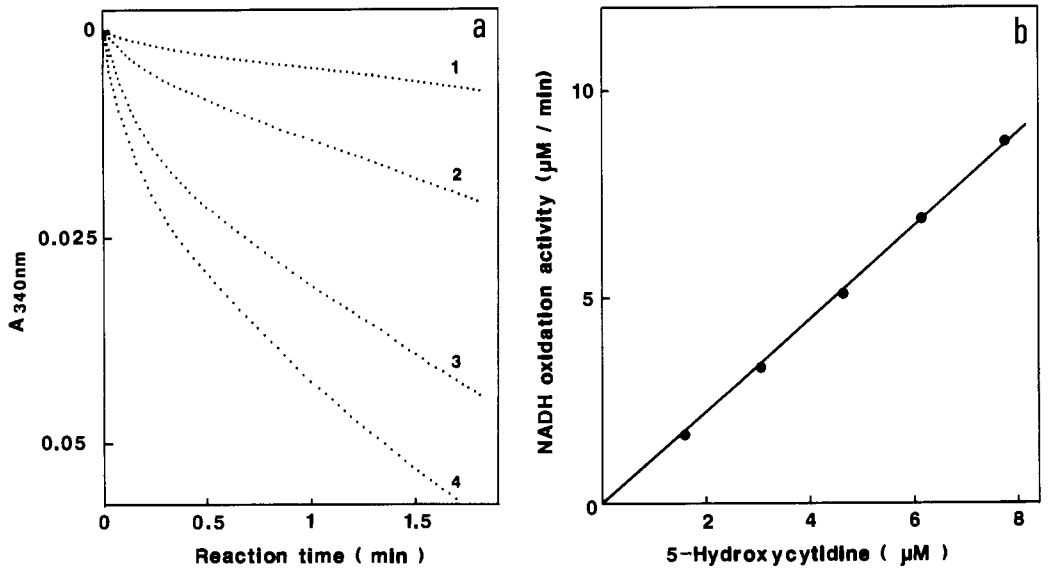


FIGURE 2: Typical progress curve (a) and activity (b) of the oxidoreduction of NADH and $K_3Fe(CN)_6$ catalyzed by 5-hydroxycytidine. The concentrations of 5-hydroxycytidine added are 0 (1), 1.54 (2), 4.64 (3), and 7.72 μM (4), respectively.

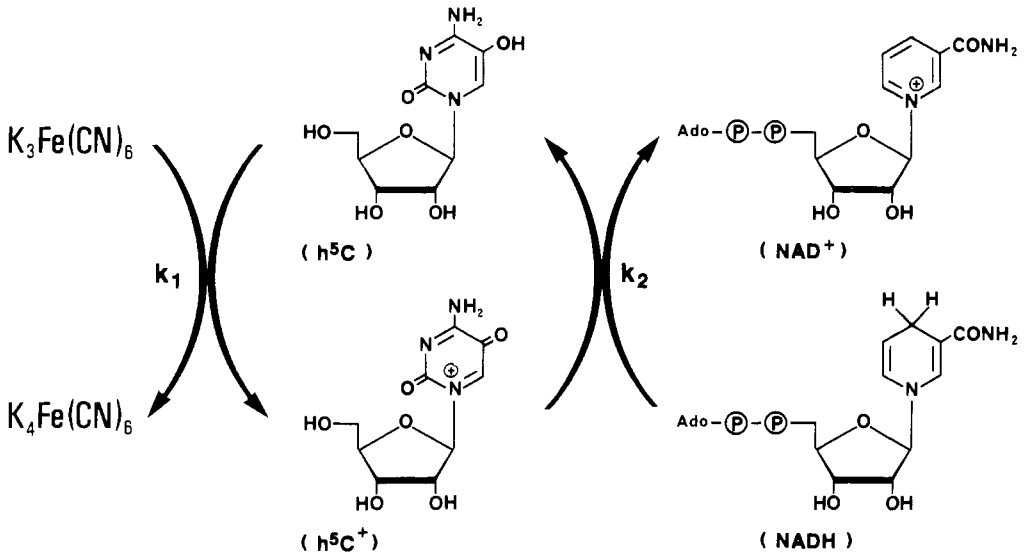
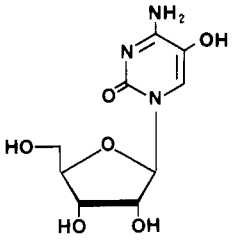


FIGURE 3: Probable scheme for the oxidoreduction of NADH and $K_3Fe(CN)_6$ catalyzed by 5-hydroxycytidine. k_1 and k_2 are the second-order rate constants of the indicated reactions.

were four resonances at 126.00, 131.08, 158.53, and 164.53 ppm, not assigned to the ribose moiety. Peaks at 126.00, 131.08, 158.53, and 164.53 ppm may possibly have been due to carbons of the 6-, 5-, 2-, and 4-positions in the pyrimidine ring, respectively. Their assignments were confirmed by nondecoupled ^{13}C NMR spectroscopy.

The above spectral data indicate that **1** may be 5-hydroxycytidine (h^5C), as was also supported by a comparison with a specimen synthesized from cytidine by the method of Visser (1968). We also confirmed that h^5C was present in RNA fractions extracted from *Torula* yeast by the method of Lis (1966).



1

To examine h^5C as an oxidoreduction catalyst, the same assay conditions as above were used. The reactions were conducted in 0.05 M Tris-HCl buffer, pH 8.26, at 30 °C, with $K_3Fe(CN)_6$ as the oxidant and NADH as the reductant. In the absence of h^5C, NADH was reduced very slowly to NAD^+ with $K_3Fe(CN)_6$. But following its addition, the oxidation was quite rapid. For example, the rate of NADH oxidation increased by factors of approximately 4, 12, and 19 at concentrations of 1.54, 4.64, and 7.72 μM h^5C, respectively. A time course of the oxidation of NADH with $K_3Fe(CN)_6$ in the presence of h^5C is shown in Figure 2a. NADH oxidation increased linearly with the concentration of h^5C (Figure 2b). NAD^+ was formed as an oxidation product as detected by HPLC on an ODS column. The conversion of $K_3Fe(CN)_6$ to $K_4Fe(CN)_6$ was confirmed from the decrease in absorption at 420 nm. h^5C is thus shown to catalyze the oxidoreduction of NADH and $K_3Fe(CN)_6$. The pH optimum for the oxidoreduction catalyzed by h^5C was pH 8.26.

A probable scheme for oxidoreduction catalyzed by (h^5C) is shown in Figure 3. h^5C was oxidized by $K_3Fe(CN)_6$ to give an oxidized form (h^5C^+) having *p*-quinoid and quaternary salt structures in the pyrimidine ring moiety, simultaneously. h^5C^+ with a quaternary salt similar to NAD^+ may be easily reduced by NADH to form h^5C, in which NADH is oxidized to NAD^+ simultaneously. The chemical structure of h^5C^+ was not determined, but a product from a reaction of h^5C with $K_3Fe(CN)_6$ was confirmed by HPLC on an ODS column.

The kinetics of oxidoreduction (Yomo et al., 1989) catalyzed by h^5C were examined as follows. In the coupled reactions, k_1 and k_2 are the second-order rate constants of the indicated reactions. The reactions in this scheme are expressed by the following:

$$d[K_3Fe(CN)_6]/dt = -k_1[h^5C][K_3Fe(CN)_6] \quad (1)$$

$$d[NADH]/dt = -k_2[NADH][h^5C^+] \quad (2)$$

$$d[h^5C]/dt = k_2[NADH][h^5C^+] - k_1[h^5C][K_3Fe(CN)_6] \quad (3)$$

$$[h^5C]_0 = [h^5C] + [h^5C^+] \quad (4)$$

where $[h^5C]$ and $[h^5C^+]$ are the concentrations of h^5C and h^5C^+, respectively. $[h^5C]_0$ shows the initial concentration of h^5C. At steady state, $d[h^5C]/dt = 0$ and eq 3 becomes

$$d[h^5C]/dt = k_2[NADH][h^5C^+] - k_1[h^5C][K_3Fe(CN)_6] = 0 \quad (5)$$

From eqs 2, 4, and 5, the following equation is obtained:

$$-d[NADH]/dt = k_1[K_3Fe(CN)_6]k_2[NADH] \times [h^5C]_0/k_1[K_3Fe(CN)_6] + k_2[NADH] \quad (6)$$

At first, we obtained k_2 by the following procedure. Under the experimental conditions of $k_1[K_3Fe(CN)_6] \gg k_2[NADH]$, eq 6 is simplified to

$$-d[NADH]/dt = k_2[NADH][h^5C]_0 \quad (7)$$

Therefore, if the concentration of $K_3Fe(CN)_6$ is large enough, the rate of oxidation of NADH becomes independent of the change in $K_3Fe(CN)_6$. k_2 was calculated to be 11.7 mM^{-1} min^{-1} with eq 7.

At steady state, eq 6 was rearranged as follows:

$$k_2[NADH][h^5C]_0/(-d[NADH]/dt) = k_2[NADH]/k_1[K_3Fe(CN)_6] + 1 \quad (8)$$

The NADH oxidation rates were measured at various initial $K_3Fe(CN)_6$. From the slope of the straight line, k_1 was

calculated to be 40.0 mM^{-1} min^{-1} with eq 8.

k_{cat}/K_m (38.9 mM^{-1} min^{-1}) for $K_3Fe(CN)_6$ was higher than that (19.1 mM^{-1} min^{-1}) for NADH. k_{cat}/K_m of h^5C were much the same as that (28 mM^{-1} min^{-1}) of IVS RNA of *Tetrahymena thermophila*. But k_{cat}/K_m of h^5C was about 10^3 times less than that [11.0×10^3 for $K_3Fe(CN)_6$ and 40.4×10^3 mM^{-1} min^{-1} for NADH, respectively] of diaphorase (EC 1.8.1.4) from *Clostridium kluyveri*. It is of interest that even a small nucleosidic catalyst, h^5C, exhibited the same catalytic efficiency as the large RNA consisting of approximately 400 nucleotides.

$FeCl_3$, cytochrome *c*, ferredoxin, 2,6-DCIP, and methylene blue were examined as oxidants in oxidoreduction catalyzed by h^5C. $FeCl_3$, 2,6-DCIP, and methylene blue could not function as oxidants for NADH oxidation catalyzed by h^5C. Cytochrome *c* and ferredoxin, each consisting of iron and protein, had an effect on NADH oxidation catalyzed by h^5C as oxidants. However, their activities were one hundredth that of $K_3Fe(CN)_6$, thus indicating the most efficient oxidant for NADH catalyzed by h^5C to be $K_3Fe(CN)_6$.

α-NADH, β-NMNH, β-NADPH, β-NHypDH, and 3-AcPyADH were examined as reductants in oxidoreduction catalyzed by h^5C. β-NMNH, β-NADPH, and β-NHypDH had the same activity as β-NADH, or less. However, α-NADH (71%) and 3-AcPyADH (39%) were less active than β-NADH as reductants. In a protein enzyme (diaphorase) system, β-NADH reduced $K_3Fe(CN)_6$ 14 times more rapidly than α-NADH. On the contrary, α-NADH generally reduces different oxidants 10 times more rapidly than β-NADH in a nonenzymatic chemical system (Hajdu & Sigman, 1977). The preference for α- and β-anomers in our system is different from both enzymatic and chemical systems. The enhancement for β-NADH in our system may be due to a specific noncovalent interaction between h^5C and the β-anomer.

To date, a great number of biocatalysts including more than 2000 species of protein catalysts (enzymes) (Dixon & Webb, 1979) and a dozen RNA catalysts (ribozymes) (Cech & Bass, 1986) have been isolated from natural sources. These biocatalysts can be classified, on the basis of constitutional components, as type I (RNA), type II (RNA–small RNA), type III (RNA–protein), type IV (protein), and type V (protein–small RNA). Type I includes ribozymes such as group I RNA IVS (Cech, 1988), group II RNA IVS (Michel et al., 1989), viroids (Symons, 1989), virusoids (Symons, 1989), and satellite RNA (Symons, 1989) that requires only an inorganic ion such as magnesium ion for catalytic activity. Type II includes an RNA containing 5-hydroxycytidine, a ribozyme with oxidoreduction activity as described above, which requires a small RNA-related compound such as NAD^+ for the expression of catalytic activity. Type III includes ribonuclease P (Guerrier-Takada, 1983), spliceosomes (Choi et al., 1986; Blumenthal & Thomas, 1988), ribosomes (Nomura, 1973; Wittman, 1977), and telomerases (Greider & Blackburn, 1987) which require proteins for catalytic activity. Type IV includes most of hydrolases (Dixon & Webb, 1979) such as sulfatases, phosphatases, and ribonucleases that do not require any cofactor for their catalytic activities. Type V includes oxidoreductases, transferases, lyases, isomerases, and ligases that require cofactors (coenzymes) consisting of small RNA-related compounds such as NAD^+, $NADP^+$, FAD, CoA, and ATP for catalytic activity (Dixon & Webb, 1979).

The finding of RNA catalytic activity of RNA molecules has prompted speculation that an "RNA world" (Gilbert, 1986), in which RNA both provides catalysis and stores information, predates life by protein catalysts. That an RNA can act on oxidoreduction indicates RNAs to possibly have possessed the ability of self-replication and metabolism prior to the involvement of proteins. Types I and II catalysts may first have evolved at an early stage of evolution, developed into type III catalysts following construction of translation systems, and subsequently evolved to type IV and type V catalysts.

Research is presently being conducted to provide greater clarification of the structure of h^5C and its physiological roles in *Torula* yeast RNAs. h^5C may possibly be linked to nucleotides through phosphodiester bonds. Studies on catalytic RNAs with metabolizing function contribute to the elucidation of RNA function and roles. 5-Hydroxyuracil was isolated as a major pyrimidine product from the hydrolysate of HCN oligomers (Ferris et al., 1978). This may suggest 5-hydroxypyrimidine derivatives such as 5-hydroxycytidine and 5-hydroxyuridine may have been present on the primitive earth.

Acknowledgments

We express our appreciation to Mr. T. Yomo for his comments on the kinetics of 5-hydroxycytidine and to Ms. M. Oku for obtaining the FAB-MS.

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