A NOVEL UDP-SUGAR, UDP-3-KETOGLUCOSAMINE OR UDP-4-KETOGLUCOSAMINE, FROM BOVINE HEART MUSCLE REDUCES METMYOGLOBIN WITH NAD(P)H

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Summary
3-Ketoglucose and similar ketosugars have been identified in microorganisms only and little is known about their functions. UDP-sugars are widely found as an intermediate in sugar metabolism in living organisms. Yet what role UDP-sugars play, or whether they play a direct role in metabolism, is still unknown. UDP-sugars were isolated and purified from bovine heart muscle, and a UDP-sugar fraction capable of NAD(P)H-dependent catalytic reduction of metmyoglobin was detected. Subsequent identification revealed that the active UDP-sugar was UDP-3- or UDP-4-ketoglucosamine. These compounds were purified from bovine cardiac muscle by ultrafiltration, anion-exchange column chromatography and reverse-phase chromatography. They were further characterized by determination of their chemical reducing activity, by comparison with synthetic UDP-3- or UDP-4-ketoglucosamine standards using high-performance liquid chromatography, by estimation of molecular mass using fast atom bombardment mass spectrometry, and by Fourier transform infrared microspectroscopy and electron probe microanalysis. The results suggest that UDP-3- or UDP-4-ketoglucosamine reduces metmyoglobin in bovine cardiac muscle. It is important that the reducing activity displayed by this ketosugar is not the effect of UDP-3- or UDP-4-ketoglucosamine alone but depends on NAD(P)H. In other words, this action of UDP-3- or UDP-4-ketoglucosamine is catalytic.

Key words: UDP-sugar, amino-sugar, myoglobin, oxidation–reduction, catalysis, heart muscle.

Introduction
Myoglobin is a hemoprotein used for oxygen storage in the cardiac and skeletal muscles of vertebrates. After binding oxygen, it becomes oxymyoglobin, which can then auto-oxidize to form metmyoglobin, consequently losing its oxygen-binding activity (Gotoh and Shikama, 1974, 1976). Auto-oxidation also affects hemoglobin, the hemoprotein in erythrocytes, in a similar manner. Indeed, investigation of a hereditary disease in which auto-oxidized hemoglobin accumulates in erythrocytes led to the discovery of methemoglobin (Gibson, 1993).

Metmyoglobin and methemoglobin are chemically reduced by ascorbic acid, glutathione, the tryptophan metabolite 5-hydroxyanthranilic acid (Goda et al. 1977) and other intracellular agents as well as by two enzymatic reductases. After reduction, both hemoproteins recover their oxygen-binding capacities. One type of NADH-dependent reductase specifically reduces both metmyoglobin (Hagler et al. 1978; Levy et al. 1985; Matsui et al. 1975; Rossi-Fanelli et al. 1957) and methemoglobin (Agar and Harley, 1972; Hultquist and Passon, 1971; Kuma et al. 1972a,b; Passon and Hultquist, 1972; Scott and McGraw, 1961; Scott et al. 1964; Sugita et al. 1971; Vetrella et al. 1971) via the hemoprotein cytochrome b5. Reductases of this general type have been isolated and purified from a variety of mammalian species.

The other type of reductase is flavin- and NADPH-dependent as well as methemoglobin-specific (Nagai et al. 1980; Yubisui et al. 1977, 1979). In addition to erythrocytes and skeletal muscle, NADH-cytochrome b5 reductase is also found in liver and leukocytes, and it is believed to catalyze a wide range of reactions (Kuma et al. 1976; Leroux and Kaplan, 1972). Its enzyme kinetics and reaction mechanism are the subjects of continuing interest (Livingston et al. 1985; Tomoda et al. 1979). Other redox-active agents, such as flavins (Stepuro et al. 1992) and phenazine methosulfate (Kajita et al. 1970), are also known to mediate electron transfer between NAD(P)H and methemoglobin. In addition, cytochrome c, another hemoprotein component of the respiratory chain, has been reported to be directly involved in oxidation-reduction and is

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thought to be important for the auto-oxidation of myoglobin (Wu et al. 1972).

3- and 4-ketosugars occur naturally in microorganisms. For example, two 3-ketoglycosides have been isolated from Alcaligenes faecalis (Bernards and Deley, 1960), TDP-4-keto-6-deoxyglucose is found in Escherichia coli (Okazaki et al. 1962) and 3-ketoglucone, 3-ketosucrose and UDP-3-ketoglucone occur in Agrobacterium tumefaciens (Wu et al. 1972). These are thought to be produced in parallel with the transport of glycosides, sucrose and glucose along conventional pathways of sugar metabolism. They may also be derived from these sugars as intermediates during active transport in cells (Chern et al. 1976; Miyama and Fukui, 1975). Ketosugars are formed enzymatically inside microorganisms and, in the case of glucose, 3-ketoglucose reductase performs this function (Hayano et al. 1973).

UDP-keto-hexoses, UDP-3-ketoglucose and UDP-4-ketoglucose exist as intermediate products of the isomerization between UDP-glucose and UDP-galactose. The enzyme catalyzing this reaction, UDP-galactose 4¢-epimerase, has been purified from E. coli and from yeast (Davis and Glaser, 1971; Maitra and Ankel, 1971, 1973). These studies also suggest that ketosugars have reducing activity similar to that of ascorbic acid in vivo. In addition, because ketosugars display specific absorption maxima under alkaline conditions, they can be detected spectroscopically (Fukui and Hayano, 1969). Their existence in metazoans has not been previously established, however.

UDP-glucosamine and its isomer UDP-galactosamine are present in much smaller amounts than are other UDP-sugars. They were detected only when excess amounts of galactosamine were present despite the fact that enzyme groups with UDP-glucose substrates are able to catalyse the biosynthesis of UDP-hexosamines in vitro (Kepppler and Decker, 1969; Maley, 1970; Maley and Maley, 1959; Tarentino and Maley, 1976; Weckbecker and Keppler, 1982). Here, we report that UDP-3- or UDP-4-ketoglucosamine purified from bovine heart chemically reduces metmyoglobin from cardiac muscle.

Materials and methods

Preparation of a low-molecular-mass fraction from cardiac muscle

One bovine heart, obtained from a local abattoir, was used for extraction. Fat and connective tissue were removed; the remaining heart weighed approximately 1.2 kg. The heart was minced, and the tissue fragments were soaked in 1.2 l of 30 mmol l⁻¹ Tris·HCl buffer containing 0.1 mol l⁻¹ KCl and 50% methanol (both by volume, pH 8.0) and then homogenized in a blender. After straining the mixture through gauze, the filtrate was centrifuged (10 000 g, 30 min, 4°C). The supernatant was evaporated at 50 °C and, after removal of the methanol, the residual liquid was filtered progressively through membrane filters that excluded material with a molecular mass greater than 10 kDa (YM10 Diaglo membrane, Amicon, division of W. R. Grace & Co., USA), greater than 3000 Da (YM3) and greater than 1000 Da (YM1) to obtain a low-molecular-mass fraction (stirred ultrafiltration cell 8400, internal diameter 76 mm, Amicon). After lyophilization, ethanol and acetone (1:1) were added, and the pellet was washed. Lipids were removed from the supernatant, and solutes were precipitated and dried. Then, 0.9 mol l⁻¹ perchloric acid was added, and the mixture was stirred thoroughly and centrifuged (at 2000 g for 10 min) to collect the supernatant. The solution was neutralized with KHCO₃ and centrifuged (at 2000 g for 10 min), and the supernatant was collected and passed through a 500 Da ultrafiltration membrane (Amicon YC05). The fraction containing molecules with a molecular mass greater than 500 Da was subjected to further rinsing to desalt and condense it by ultrafiltration. After purging of the low-molecular-mass compounds, distilled water was again added and the process was repeated. The resulting fraction, approximately 50 ml of material of molecular mass 500–1000 Da, was used as the crude sample in the subsequent high-performance liquid chromatography (HPLC) isolation and purification steps. Scanning of the ultraviolet spectrum of the resulting sample (U-3200, Hitachi) confirmed that the peak was in the neighborhood of 260 nm, corresponding to nucleic acids and related substances. The crude sample was lyophilized and stored at −20°C.

Purification by HPLC

Approximately 10 mg of the crude sample was dissolved in 10 ml of distilled water. A weak anion exchange column (DEAE-2SW, 7.8 mm x 30 cm, Tosoh, Japan) was used as a separator; the pump was a model NPL-3300 (Nihon Seimitsu Kagaku Co., Japan), and the detector was an S-310A model II (Soma Optics, Japan). Specific optical absorbance of uridine nucleotides was detected at 262 nm. The specimen was injected in 0.5 ml samples, and the eluate was fractionated and collected separately for each peak; the metmyoglobin reducing activity of each peak was measured. The fraction showing reducing activity was desalted by ultrafiltration through a membrane excluding molecules with a molecular mass of 500 Da or more. The sample was then eluted in methanol using a reverse-phase chromatography column (ODS-80TM, 7.8 mm x 30 cm, Tosoh) to isolate the target substance. The purified product was lyophilized and stored at −20°C.

Synthesis of UDP-hexosamines

Uridylyltransferase (4.5 units; one unit forms 1.0 μmole UDP-galactose and glucose-1-phosphate per minute at pH 8.0, 25 °C; EC 2.7.7.12, Sigma) and 2 mg of UDP-galactose (Sigma) were added to 5 ml of 75 mmol l⁻¹ Tris·HCl buffer, 150 mmol l⁻¹ triethanolamine and 10 mmol l⁻¹ cysteine (pH 7.4). The reaction mixture was shaken gently and preincubated at 37°C for 10 min. Next, 10 mg of glucosamine 1-phosphate (Sigma) was added, and the solution was shaken gently and incubated at 37°C for 40 min. The reaction was stopped by immersion of the tube in a water bath at 95°C for
3 min. The solution was centrifuged at 11 000 g for 4 min, and the supernatant was collected for filtration through a 0.2 μm pore membrane. During this process, approximately 80% of the UDP-galactose was converted to UDP-glucosamine (Kepler and Decker, 1969; Maley, 1970; Tarentino and Maley, 1976; Weckbecker and Kepler, 1982). UDP-galactosamine synthesis was performed in a similar manner using UDP-glucose (Sigma) and galactosamine 1-phosphate (Sigma) as the substrate.

**Synthesis of UDP-3- or UDP-4-ketoglucosamine**

Ketosugars were synthesized by an improvement of the method employing UDP-galactose 4′-epimerase (EC 5.1.3.2, Sigma) to obtain enzymatic isomerization intermediates (Fukui and Hayano, 1969; Maitra and Ankel, 1971, 1973). UDP-galactose 4′-epimerase (1 unit) was mixed with 3 μmol of UDP-glucosamine or UDP-galactosamine in 30 mmol l−1 Tris-HCl buffer, pH 8.0 (1 unit of UDP-galactose 4′-epimerase will convert 1.0 μmole UDP-galactose to UDP-glucose per minute at pH 8.8, 25 °C). After incubation for 2 h at 37 °C, the mixture was heated at 95 °C for 2 min and centrifuged at 8000 g for 4 min. The supernatant was retained. To detect ketosugars, changes in specific optical absorbance at 350 nm that accompany the decomposition of ketosugars in an alkaline environment (Fukui and Hayano, 1969) were measured. The synthesized products were desalted by ultrafiltration, lyophilized and stored at −20 °C.

**HPLC of UDP-hexosamines**

A method for specific analysis of amino sugars was developed as follows. Labeling of amino sugars with phenylisothiocarbamoyl (PTC) was carried out as described (Wako Chemical Co., Japan). First, approximately 100 nmol of purified sample was collected in a microtube. The amount of UDP-sugar was measured from the absorbance at 262 nm. The bases, nucleosides and nucleotides used as standards were all from Sigma Chemical Co. (USA).

**Structural determination by spectral analysis**

To measure the molecular mass of the sample purified by HPLC, positive-ion fast atom bombardment (FAB) mass spectrometry was performed using a JMS-DX302 mass spectrometer (JEOL Ltd, Japan). The direct inlet method was used, and the scope of measurement was set at the molecular mass range 50–700 Da, with a scan speed of 7.39 s, an ion voltage of 1.4 kV, using argon gas, an FAB gun voltage of 3 kV and an emission current of 20 mA. The matrix was glycerine or magic burette (Gaskell, 1986). Functional groups were identified using a Fourier transform infrared (FT-IR) microspectroscopic analyzer (Hitachi-Nicolet) (Okada et al. 1990). Elements other than C and N were measured using electron probe microanalysis (EPMA) (JXA733, JEOL) with an energy-dispersive X-ray detector system (TN5400, Noran Instruments) (Shinoyama, 1994).

**Measurement of metmyoglobin reducing activity**

The reducing activity of the fractionated samples and of
synthetic UDP-3- or UDP-4-ketoglucosamine were measured using metmyoglobin. Commercially available myoglobin from bovine heart muscle (Sigma) is already partly oxidized, and most has become metmyoglobin. The method of measurement was based on that for metmyoglobin reductase activity (Hagler et al. 1978). To adjust the metmyoglobin concentration to 0.5 mmol l\(^{-1}\), the metmyoglobin was dissolved in 25 mmol l\(^{-1}\) phosphate–citrate buffer, pH 7.0, containing 0.5 mmol l\(^{-1}\) EDTA. β-NADH or β-NADPH was dissolved in distilled water to adjust the concentration to 5 mmol l\(^{-1}\). The nucleotide concentration in the samples was calculated from the molar absorption coefficient for uridine nucleotides at 262 nm (\(\varepsilon=10\,000\)). Measurement buffer (390 μl), metmyoglobin (50 μl) and sample (50 μl) were added to a 1 cm thick 1 ml cuvette and stirred briefly. Precisely 10 μl of NAD(P)H was then added and, after stirring, changes in absorbance were measured at 580 nm, the absorbance maximum of reduced myoglobin (Brown et al. 1962). Measurements were conducted over 30 min.

**Results**

**HPLC of the UDP-sugar fraction with metmyoglobin reducing activity**

The dry mass of the total crude extract from one bovine heart was approximately 1 g. Fig. 1A is a chromatogram showing separation of the crude sample on a DEAE column. The shaded peak where the retention time was 7.2±0.2 min (mean ± s.d. of 20 samples) is where NAD(P)H-dependent metmyoglobin reduction activity was observed. The absorbance maximum at 262 nm observed after separation suggests that this is a uridine nucleotide. Examination of different uridine nucleotides revealed the existence of UDP-glucose and UDP-N-acetylglucosamine in the peak fraction. These UDP-sugars had no metmyoglobin reducing activity, however. The peak indicated by the asterisk is UDP-hexosamine.

Fig. 1B is a chromatogram of the DEAE-separated fraction further purified by passage through an ODS column. The shaded peak where the retention time was 4.9±0.3 min (mean ± s.d. of 20 samples) is where NAD(P)H-dependent metmyoglobin reduction activity was observed. The peak after 20 min of elution includes UDP-glucose and UDP-N-acetylglucosamine. The active fraction was found to be a single peak when a size-exclusion column was used. Consequently, isolation and purification were considered to be complete at this point. Table 1 summarizes the purification of UDP-sugar showing metmyoglobin reducing activity. From these data and the molar extinction coefficient at 262 nm, 0.12±0.03 nmol g\(^{-1}\) wet tissue of metmyoglobin reducing factor was calculated to be present. Taking into consideration the recovery rates of the purification steps, the crude extract...
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Concentration is 3.6 μmol l\(^{-1}\). This is a concentration sufficient to reduce metmyoglobin (see Fig. 6B).

**Identification of 3- and 4-ketosugars**

Fig. 2 shows that changes in the ultraviolet spectrum specific for 3- and 4-ketosugars under alkaline conditions are observed at around 300 nm in the purified sample. An acid-treated control spectrum is also depicted. Similar results were obtained with synthetic UDP-3- or UDP-4-ketoglucosamine.

**HPLC detection of UDP-3- or UDP-4-ketoglucosamine**

Fig. 3 is a chromatogram produced using a reverse-phase column after PTC labeling of amino sugars in the purified sample. The ketosugar, whose retention time is 22.4±0.4 min (mean ± S.D. of 4 samples), is clearly distinguished from the UDP-hexosamine. This method demonstrates that UDP-glucose and UDP-N-acetylglucosamine are not labeled by PTC and are therefore rapidly eluted. Consequently, separation of UDP-hexosamines from their derivatives is very efficient. PTC-UDP-hexosamine and PTC-hexosamine are degradation products.

**Characterization of the isolated sample**

These findings are in agreement with the observed results from column chromatography and the synthetic UDP-3- or UDP-4-ketoglucosamine, which also showed metmyoglobin reducing activity. HPLC and biochemical analyses of hydrolysates (Table 2) also indicated that the isolated sample was a derivative of UDP-hexosamine.

Fig. 4A,B shows spectral analysis data from the isolated sample. Fig. 4A is a FAB mass spectrogram. Pseudomolecular ions ([M+H]\(^{+}\)) were detected at a nominal \(m/z\) of 630. The presence of sodium was observed at \(m/z\) values of 652 and 674, a shift of 22 from the molecular ion. The molecular mass of the sample therefore appears to be 629 Da. The molecular mass

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**Table 1. Summary of the purification steps for UDP-ketosugar from bovine heart muscle**

<table>
<thead>
<tr>
<th>Step</th>
<th>Procedure</th>
<th>Volume (ml)</th>
<th>(A_{262})</th>
<th>Total (A_{262})</th>
<th>Specific activity (nmol min(^{-1}) absorbance unit(^{-1}))</th>
<th>(A_{262}) yield (%)</th>
<th>Recovery (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Crude</td>
<td>50</td>
<td>3.40</td>
<td>170</td>
<td>0.31</td>
<td>100</td>
<td>100</td>
</tr>
<tr>
<td>2</td>
<td>DEAE</td>
<td>32.9</td>
<td>1.62</td>
<td>53.3</td>
<td>0.57</td>
<td>31.4</td>
<td>58</td>
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<td>ODS</td>
<td>2.7</td>
<td>0.54</td>
<td>1.46</td>
<td>1.23</td>
<td>0.86</td>
<td>3.3</td>
</tr>
</tbody>
</table>

Metmyoglobin reduction activity was measured as described in Materials and methods. \(A_{260}\), absorbance at 260 nm.

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Fig. 2. Ultraviolet spectra of the purified sample from bovine heart muscle: in water (solid line), in 0.1 mol l\(^{-1}\) HCl (dashed line), in 0.1 mol l\(^{-1}\) NH\(_3\) (dotted line) and in 0.1 mol l\(^{-1}\) NaOH (dot–dashed line).

Fig. 3. UDP-sugar analysis using reverse-phase phenylthiocarbamoyl-labeled high-performance liquid. Linear gradient elution with 60 mmol l\(^{-1}\) acetate buffer and 6% to 24% (v/v) acetonitrile was performed for 30 min at a constant flow rate (1.0 ml min\(^{-1}\)). The column was heated at 40 °C. The eluate was monitored at 254 nm, detected by a flow cell with a light pass length of 1 mm.
An odd number because uracil has two nitrogen atoms and the amino-sugar has one.

Fig. 4B is an FT-IR spectrum showing specific absorbance curves of the UDP-sugar. The findings of elemental analysis by EPMA indicate the presence of P and Na. The component elements of the UDP-sugar are therefore likely to be C, H, O, N, P and Na. These findings identify the isolated sample as UDP-3- or UDP-4-ketoglucosamine. Fig. 5 is a diagram of its molecular structure. Phosphate groups are shown in the electrolytically dissociated state. The molecular mass is that of the free acid. The three-sodium salt has a molecular mass of 629 Da.

### Kinetics of metmyoglobin reducing activity

Fig. 6A shows the metmyoglobin reducing activity of the isolated sample (7.6 μmol l⁻¹). It should be noted that UDP-3- or UDP-4-ketoglucosamine do not work alone as reducing agents. Indeed, they have the opposite effect, promoting the oxidation of myoglobin. Reducing activity is found only in the presence of NADPH (0.20 nmol oxidised in 30 min).

Fig. 6B plots changes in the rates of metmyoglobin reducing activity.
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Uridine diphosphate 4-ketoglucosamine (UDP-4-ketoGlcN)

Uridine diphosphate 3-ketoglucosamine (UDP-3-ketoGlcN)

Fig. 5. Molecular structure of UDP-4-ketoglucosamine and UDP-3-ketoglucosamine.

Fig. 6. (A) Metmyoglobin reducing activity of UDP-3- or UDP-4-ketoglucosamine: with UDP-3- or UDP-4-ketoglucosamine and NADPH (solid line), with NADPH only (dotted line), with UDP-3- or UDP-4-ketoglucosamine only (dot–dashed line) and control (dashed line). A cuvette with a light pass length of 1 cm was used. (B) Effect of varying the amount of UDP-3- or UDP-4-ketoglucosamine on metmyoglobin reducing activity. Metmyoglobin and NADH concentrations in all assays were 50 µmol l⁻¹ and 100 µmol l⁻¹, respectively. (C) Effect of varying metmyoglobin concentration on metmyoglobin reducing activity. UDP-3- or UDP-4-ketoglucosamine and NADH concentrations in all assays were 5.4 µmol l⁻¹ and 100 µmol l⁻¹, respectively. (D) Lineweaver–Burk plot prepared from the data in C. V, rate of reduction of metmyoglobin. All experiments were conducted in duplicate.
activity resulting from varying the concentration of UDP-3- or UDP-4-ketoglucosamine. Rates were determined by determining the initial activity and calculating the molar absorption coefficient (ε=12 000) (Livingston et al. 1985) for the difference in absorbance at 580 nm between reduced myoglobin and oxidized metmyoglobin. Tests using NADPH were performed in the same way.

Fig. 6C depicts metmyoglobin reducing activity. However, in this case, metmyoglobin concentrations were varied. It also shows that the reaction approaches saturation when the metmyoglobin concentration is 200 μmol l⁻¹. Using these values and assuming that the ketosugar mimics an enzyme, a Lineweaver–Burk plot was drawn (Fig. 6D). Vmax is 0.23 μmol l⁻¹ min⁻¹ and Km is 6.25×10⁻⁵ mol l⁻¹.

Discussion

In previous studies, the occurrence of UDP-hexosamines has been demonstrated in vivo only when levels of cell-toxic galactosamine were high (Decker and Keppler, 1972, 1974; Keppler et al. 1968, 1970; Maley et al. 1966, 1968). The liver, where sugar metabolism is very active, has been the preferred organ for experimental investigation in mammals. The heart, which was used in the present study, has not been considered before. Nevertheless, cardiac and skeletal muscle are sites of active glycogen metabolism, and therefore research on sugar metabolism is very active, has been the preferred organ for experimental investigation in mammals. The heart, which was used in the present study, has not been considered before. Nevertheless, cardiac and skeletal muscle are sites of active glycogen metabolism, and therefore research on sugar metabolism is very active, has been the preferred organ for experimental investigation in mammals.

UDP-glucosamine and UDP-galactosamine are interconvertible, and this reaction is catalyzed by an isomerase. However, whether the enzyme in this case is identical to UDP-galactose 4′-epimerase, the enzyme that catalyzes UDP-hexose, is unknown. The formation of UDP-hexose involves both UDP-3-ketoglucose and UDP-4-ketoglucose as intermediates (Fukui and Hayano, 1969; Maitra and Ankel, 1971, 1973). Thus, it is possible that similar UDP-hexosamine intermediates exist and that they may be interconvertible in a manner analogous to that for ascorbic acid intermediates (Fukui and Hayano, 1969).

The present demonstration that 3- and 4-ketosugars, which hitherto had been detected only in microorganisms, also exist with significant reducing activity in higher organisms suggests that 3- and 4-ketosugars may play important physiological roles in cardiac muscle. Metmyoglobin reducing activity was catalytic and NAD(P)H-dependent. Normally, the oxidation–reduction potential of NAD(P)H in vivo is very low, so UDP-3- or UDP-4-ketoglucosamine may act as an electron carrier between myoglobin and NAD(P)H, as do flavins and phenazine methosulfate. More studies are needed to investigate further the reducing activity of UDP-3- or UDP-4-ketoglucosamine on metmyoglobin and other hemoproteins. Defining their physiological roles may well advance our understanding of oxidation–reduction reactions in vivo.

Enzymes, in vivo catalysts, are proteins. Recently, a type of RNA called ribozyme has been reported to exhibit catalytic activity during splicing and cleavage of nucleic acid (Cech and Bass, 1986; Cech, 1987). In addition, the existence of low-molecular-mass compounds, nucleosides (Yanagawa et al. 1990, 1992) and dipeptides (Shimizu, 1995a,b), exhibiting catalytic activities has also been reported. These nucleosides act as catalysts for reduction reactions. It is possible that UDP-3- or UDP-4-ketoglucosamine also acts as a catalyst for a metmyoglobin reduction pathway.

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