HYDROXYLAMINE REDUCTASE OF A HALOTOLERANT MICROCOCCUS*

M. KONO AND S. TANIGUCHI

Department of Chemistry, Faculty of Science, Nagoya University, Chikusa, Nagoya (Japan)

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SUMMARY

A hydroxylamine reductase of hemoprotein nature was prepared from a strain of halotolerant Micrococcus. The hydroxylamine reductase was shown to contain iron as its active center. Manganese ions at 0.5 m*M* concentration activate the reaction about 3 to 4 times when the activity is measured by the reduced methylene blue method; these ions possibly participate in the formation of an hydroxylamine reductase–hydroxylamine complex probably with a simultaneous valency change. The reduced form of this hemoprotein is oxidized by both oxygen and hydroxylamine, the reactions being similarly activated by manganese ions. Carbon monoxide combines with the reduced hemoprotein photo-reversibly. Pyridine hemochromogen from this enzyme has the same absorption maxima as that from mammalian cytochrome *c*. This autoxidizable and carbon monoxide-binding cytochrome *c* (its reduced α-peak is at 554 mμ) which can be obtained from the halotolerant Micrococcus is shown to be hydroxylamine reductase. A possible relationship between the chemical structure and enzyme activity was discussed.

INTRODUCTION

Hydroxylamine reductase (HdR) which catalyzes the stoichiometric reduction of hydroxylamine to ammonia was first isolated in the cell-free state from *Bacillus pumilus var.* as one of enzymes which catalyzed the "successive reduction process" from nitrate to ammonia via hydroxylamine[1]. Several papers on its chief characteristics as well as on the attempts to purify the enzyme have been already published using several strains of halotolerant bacteria[2–4].

Since 1954, pyridine nucleotide-HdR from *Neurospora crassa*[5], soy bean leaves[6], *Azotobacter agile*[7] has been reported. This group of HdR's is considered to have a physiological function in the assimilation of nitrate.

It has been noticed that successive reduction by a halotolerant Micrococcus (Strain 203) is not always reproducible; the Micrococcus grown under the present

The following abbreviations are used: hydroxylamine reductase, HdR; reduced diphosphopyridine nucleotide, DPNH; cytochrome, cyt.; reduced methylene blue, MbH_2; tris (hydroxymethyl) aminomethane, Tris; methylviologen, MV; reduced MV, MVH.

* Part of this work was read at The International Symposium of Enzyme Chemistry, Tokyo and Kyoto, 1957 (ref. 1).

conditions sometimes does not accumulate ammonia, but instead produces appreciable amounts of nitrogen gas, in either Thunberg tubes or Warburg manometers when formate or MbH_2 is used as the electron donor[8]. The Micrococcus, even when strongly denitrifying, still possesses an active HdR system. Similar observations have also been made with typical denitrifyers such as *Micrococcus denitrificans* and *Pseudomonas denitrificans*[9]. Thus the inorganic nitrogen metabolism in these denitrifying organisms may be expressed as follows:

$$NO_3^- \longrightarrow NO_2^- \overset{N_2}{\longrightarrow} \qquad NH_2OH \longrightarrow NH_3$$

It seems, therefore, probable that "HdR" may have a physiological function other than the mere reduction of hydroxylamine*.

In this paper, further purification and properties of the terminal HdR from the same Micrococcus will be reported. Furthermore its relation to the soluble, autoxidizable and CO-binding cytochrome from the same microbe[11] will also be discussed.

MATERIALS AND METHODS

Microorganism

A halotolerant Micrococcus, Strain 203, was grown aerobically in a 100 l stainless steel tank at 35° for 24 h using the following medium: 700 g of peptone, 700 g of meat extract, 7 kg of NaCl, 700 g of KNO_3, and 14 g of $MgSO_4 \cdot 7H_2O$, in 70 l of tap water, pH 7.2.

Cell-free extract

Grown cells were separated from the medium by a Sharples Supercentrifuge and washed once with 10% NaCl. The washed cells were added to distilled water where they formed a thick suspension and were lyophilized. From 70 l of medium 120 g of dry cell powder was obtained. The lyophilized bacterial powder was stable for two months at 5° in a vacuum desiccator. To prepare a crude extract, 7–8 g of the powder per 100 ml was suspended in a 10 m*M* phosphate buffer, pH 6.8. The suspension was dialyzed overnight in the cold against a large volume of the same buffer. The dialyzed suspension was centrifuged at 18,000 × *g* for 20 min at 0°. The residue was dissolved in a small amount of the same buffer and again the suspension was centrifuged at 18,000 × *g* for 20 min. These supernatant solutions were combined to give a cell-free dark-brown fluid which contained more than 85% of the HdR activity in the original suspension. This was used as the crude extract for further purification.

Calcium phosphate gel

A calcium phosphate gel was made according to the method of KEILIN *et al.*[12] with the following modification. The gel was washed eight times with distilled water until the washings were at pH 8. The gel was adjusted to contain 30 to 40 mg/ml. of dry gel. Fresh gel was used without aging and it was stable for at least one month.

Ion exchange resin

An anion-exchange resin, Dowex 2 (cross-linkage 8–10%), was treated according to the method of BOMAN *et al.*[13].

* Recently it was found that the homogeneously purified HdR from the micrococcus possibly functions as a soluble activator for the particulate nitrite reductase of the same bacterium[10].

Purification procedures

All procedures were carried out below 4° unless otherwise stated.

Acetone fractionation. 5 *M* sodium chloride and 0.2 *M* phosphate buffer, pH 6.8 were added to the crude extract (Preparation 1) to give final concentrations of 0.33 *M* and 0.02 *M*, respectively. Purified acetone which had previously been cooled in a mixture of ice and NaCl at —15° was added carefully to the extract to 40% (v/v). After standing for 15 min at —10°, the precipitate was removed by centrifugation for 10 min at 3000 × *g*. Sufficient acetone was added to the supernatant to give a 52% (v/v) solution of acetone. After 15 min at —10°, the red precipitate formed was collected by centrifugation. It was dissolved in a small volume of distilled water and lyophilized (Preparation 2).

Calcium phosphate gel treatment. 1 g of Prep. 2 was dissolved in 150 ml of 0.01 *M* acetate buffer, pH 5.5, to which about 1 g (dry weight) of calcium phosphate gel was added with stirring. Before carrying out a full-scale experiment, a trial was conducted on a small scale to determine the most favorable conditions. After 10 min the gel with the absorbed HdR was collected by centrifugation, washed twice with the original volume of 33 m*M* phosphate buffer, pH 6.8, and finally eluted twice with 0.1 *M* phosphate buffer, pH 6.8. This eluate was termed Preparation 3.

Ion exchange resin chromatography: Prep. 3 which contained a high concentration of phosphate, was dialyzed against a large volume of distilled water for several hours with mechanical stirring. Then the dialyzed and phosphate-free preparation was passed through a column of the chloride-form of Dowex 2, which had been buffered with 0.02 *M* Tris–HCl buffer, pH 7.3 according to BOMAN *et al.*[13]. Elution was carried out successively with 50 ml of 0.02 *M*, 50 ml of 0.3 *M*, 40 ml of 1.0 *M* Tris–HCl buffer, pH 7.3 and finally 100 ml of 1 *N* HCl. After the dead volume of the column had passed through the column, a 5 ml-fraction was collected in a small test tube by an automatic fraction collector. Three major protein peaks were observed and the HdR activity was found in the last peak which had been eluted by the 1.0 *M* Tris–HCl buffer. This fraction was called Preparation 4. Results of the purification are summarized in Table I.

TABLE I

SUMMARY OF PURIFICATION OF MICROCOCCUS HYDROXYLAMINE REDUCTASE

Preparation No.		*Total activity*	*Specific activity*	*Per cent recovery*
1	Crude extract	1020	4.1	100
2	Acetone fraction (40–52%)	560	17.0	55
3	Calcium phosphate gel eluate	400	130	40
4	Dowex 2 eluate	360	870	36

Electrophoretic and ultracentrifugal analyses

The purity of Prep. 4 was examined with a Hitachi electrophoretic apparatus and a Spinco Model E analytical ultracentrifuge. At least one colorless contaminating component was found in the final preparation.

Measurement of HdR activity

Reduced methylene blue method. HdR activity was measured anaerobically using MbH_2 as an electron donor as described by SATO *et al.*[14] in a final volume of 2.5 ml

of reaction mixture containing the following components*: 0.05 *M* phosphate buffer, pH 6.8; enzyme preparation; 7.5 μmoles MbH_2 (in main compartment of Thunberg tube); 6.25 μmoles hydroxylamine sulfate (in side compartment). After a given time at 37°, the decrease of the hydroxylamine was determined by the method of CSÁKY[16]. In the case of highly purified preparations (Prep. 3 or 4), 1.25 μmoles $MnCl_2$ was added as a specific activator to the main compartment.

Spectrophotometric method. The activity of Preps. 3 and 4 could also be measured spectrophotometrically. The enzyme preparation in 2.9 ml of 50 m*M* phosphate buffer, pH 6.8, was reduced by the addition of 0.1 ml of fresh 0.02 *M* sodium dithionite solution in the main compartment of a Thunberg tube-type cuvette and 0.1 ml of 10 m*M* hydroxylamine sulfate solution was placed in the side compartment. After the evacuation, the reaction could be observed to begin at room temperature by the transfer of the substrate from the side to the main compartment, followed by a change in the optical density at 420 mμ, which was later shown to be caused by a Soret band of the reduced HdR. An Hitachi electrospectrophotometer model ERB-U was employed throughout this work.

Definition of the enzyme units and specific activities

In the MbH_2 method, one unit of HdR was taken to represent the amount of enzyme required to reduce 1.0 μmole of hydroxylamine per hour. In the spectrophotometric method, on the other hand, one unit of HdR was expressed as the amount of enzyme which brought about a drop of 0.001 in the optical density at 420 mμ per min. The specific activities of these were expressed as units of activity per mg of Kjeldahl nitrogen in the enzyme preparation.

Reaction with oxygen

The activity was measured spectrophotometrically in the same way as has been described for the spectrophotometric method except that the gas phase was air without hydroxylamine.

Detection of the valency change of manganese ions during the enzymic reaction

In order to detect the possible occurrence of Mn^{3+} produced from Mn^{2+} during NH_2OH reduction, KAHANE's formaldoxime–Mn^{3+} complex method[17] utilizing the wine red color of the formaldoxime–Mn^{3+} complex was applied. In this case, the composition of the standard reaction mixture was modified by replacing MbH_2 and phosphate buffer, pH 6.8 with 2 μmoles MVH (reduced with Mg powder) and the same buffer, pH 8.0 in a total volume of 2.5 ml, respectively. In addition, 0.2 ml of 10-times diluted formaldoxime reagent (a mixture of hydroxylamine and formaldehyde) and 0.3 ml of 0.07 *M* NaOH solution were placed in the side tube together with the hydroxylamine solution added as the substrate. The Thunberg tube should be evacuated as completely as possible, since any remaining oxygen could possibly oxidize Mn^{2+} to Mn^{3+} under these conditions. After the violet color of the MVH was removed by the hydroxylamine reduction, the occurrence of a pink color complex

* Several halophilic or halotolerant enzymes the optimum salt concentrations of which are unusually higher than those of ordinary enzymes have been reported from this organism[15]. HdR, however, is neither halophilic nor halotolerant and no extra salt was added to the reaction mixture when the activity was measured by this method. However, this is not the case in the spectrophotometric method.

was observed. Under the above conditions, the MVH was shown to donate enzymically its electron to hydroxylamine and the formaldehyde was not found to affect the HdR activity. The Thunberg tube-type cuvette was employed to determine the Mn^{3+} produced and the wine red color developed was measured by its optical density at 480 mμ.

Absorption spectra

To determine the adsorption spectra of the reduced form, the enzyme was reduced anaerobically by a small amount of sodium dithionite. When the different spectra were obtained, the differences in the optical densities were calculated (reduced minus oxidized) and plotted against the wave-length.

Protein estimation

Protein was determined colorimetrically essentially by the method of LOWRY *et al.*[18].

Chemical analysis of metals

Iron, manganese and molybdenum were determined by the methods described by SANDEL[19], KIYOTA *et al.*[20] and SANDEL[21], respectively.

RESULTS

Effect and modes of action of manganese ions and Michaelis constant on substrate

It was often observed that dialysis of Prep. 3 brought about a marked decrease in the enzymic activity. Various metal ions were added to the dialyzed enzyme preparations and it was found that only $MnCl_2$ at a final concentration of 0.5 m*M* restored the activity (Table II).

TABLE II

ACTIVATION OF DIALYZED MICROCOCCUS HYDROXYLAMINE REDUCTASE BY METAL IONS

Prep. 3 was used as the enzyme preparation.

Preparation		*Specific activity*	*Per cent activation**
Non-dialysed preparation		117	
Dialyzed against distilled water for 15 h at 4°		45	
After preincubation for 30 min at 4° with 0.05 *M* phosphate buffer pH 6.8 and 0.5 m*M*** of:	$MnCl_2$	140	+210
	$MgSO_4$	33	— 27
	Na_2MoO_4	45	0
	MoO_3	50	+ 11
	$FeSO_4$	40	— 11
	$FeCl_3$	48	+ 7
	Na_2WO_4	45	0
	$NiCl_2$	41	— 9
	$CoCl_2$	43	— 4
	$ZnCl_2$	0	—100

* Percent relative to dialyzed preparation.

** The same level of each metal ion concentration was also maintained in the reaction mixture of the standard assay using reduced methylene blue.

The fact that the reproducible value of Michaelis constant (K_m) had not been obtained from the LINEWEAVER–BURK's reciprocal plot for the HdR–hydroxylamine complex led the authors to examine the kinetic relationship between the substrate and $MnCl_2$ concentration.

Curve A in Fig. 1 showing the LINEWEAVER–BURK's plot obtained from the substrate saturation data in the presence of 0.2 mM $MnCl_2$ in the MbH_2 method does not give a clear straight line, especially in the ranges of both the higher and the lower substrate concentrations. This suggests that the $MnCl_2$ concentration required for the maximal activity may vary with the substrate concentration. In fact, a $MnCl_2$ concentration approximately one-fifth of that of hydroxylamine was found to be optimal for the activity and using a constant $MnCl_2/NH_2OH$ molar ratio of 1/5 a smooth straight line (B in Fig. 1) was obtained indicating a definite K_m value of $8.1 \cdot 10^{-4}$ M. These results show that Mn ion as a specific activator can profoundly affect the substrate saturation curve suggesting the possible role of the Mn ions in the formation of the HdR–hydroxylamine complex during the hydroxylamine reduction.

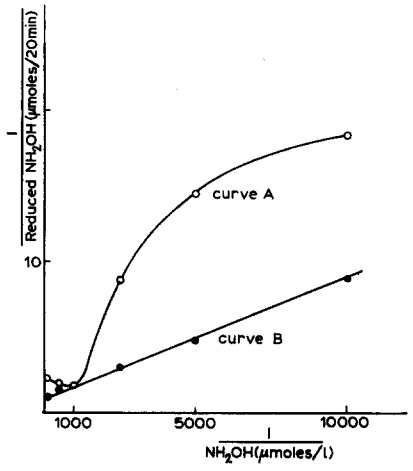


Fig. 1. The effect of NH_2OH and Mn ion concentrations on the reaction rate. Standard assay conditions using reduced methylene blue: curve A, in the presence of 0.2 mM $MnCl_2$; curve B, in the presence of $MnCl_2$ of concentration one-fifth that of NH_2OH.

However, this possibility does not always rule out that in which the Mn ions play an active role in the electron transfer undergoing alternate oxido-reduction. Therefore, the reaction mixture was examined for Mn^{3+} by the formaldoxime method[17]. The violet color of the MVH gradually changed to reddish violet and after the disappearance of the violet color of the MVH the development of clear wine red color could always be easily observed. In a boiled enzyme system, only a very pale orange color could be observed immediately after the decoloration of MVH by gentle shaking in the open air, since no enzymic disappearance of MVH occurred. Owing to the chemical autoxidation of Mn^{2+}, however, this was soon followed by a change to a deep wine red color. When Mn^{2+} was omitted from the complete system, the observed color of the complex after the enzymic decoloration of MVH was very pale orange. Omission of the reduction or MV also gave a pale color presumably due to the slight formation of MVH by formaldehyde. When formaldehyde and NaOH solution were

separated from the other components during the incubation and when they were added immediately after the MVH was exhausted, no wine red color could be observed.

These qualitative observations seem to indicate a possible valency change between Mn^{2+} and Mn^{3+} even though the pH condition (8.0) was optimal only for development of the wine-red complex and not for HdR activity. The amount of Mn^{3+} captured as the Mn^{3+}–formaldoxime complex was roughly estimated to be several % of the $MnCl_2$ initially added.

Metal analyses

HdR has long been considered to be one of heavy metal enzymes, since typical heavy metal reagents such as KCN, NaN_3, CO, α,α′-bipyridyl, 8-OH-quinoline and *o*-phenanthroline exert marked inhibitory effects on HdR activity. Therefore, using the partially purified enzyme preparations (Prep. 2 and 3), an attempt was made to analyze the metal constituents by chemical means. Of the three metals determined, only iron was found in dialyzed Prep. 3 in significant quantities (Table III).

TABLE III

METAL CONTENTS IN MICROCOCCUS HYDROXYLAMINE REDUCTASE PREPARATION

Enzyme preparation	*Metal contents (μg/mg N)*					
	Fe		*Mn*		*Mo*	
	Prep. 2	*Prep. 3*	*Prep. 2*	*Prep. 3*	*Prep. 2*	*Prep. 3*
Before dialysis	2.24	2.03	0.42	2.10	0.00	0.00
After dialysis*	1.05	2.03	0.14	0.00	—	—

* Dialysis was carried out at 4° for 24 h against deionized water.

This observation confirms the results of the inhibition experiments[2,3] which suggested the presence of iron in HdR. Manganese, on the contrary, which was present in undialyzed Prep. 3, completely disappeared on dialysis. This fact is particularly significant, since in highly purified preparations, Mn ions must be added to obtain full activity of HdR (Table II). Molybdenum was not found in any of the preparations.

Absorption spectra and cytochrome nature of HdR

Spectrophotometric analysis of Prep. 4 showed that HdR had the typical absorption spectra of a cytochrome (Fig. 2).

In the oxidized form, two bands were seen at 405 mμ and 635 mμ, while in the reduced form, three bands were found at 420 mμ, 521 mμ, and 554 mμ, two of which had shoulders at 434 mμ and 548 mμ. The data presented in Table IV show that these peaks are different from those of two types of cyt. b_4, types I and II[22] which are present in abundance in this halotolerant bacterium.

In fact, these two types of cyt. b_4 were shown to have no HdR activity by both MbH_2 and spectrophotometric methods*.

The method for preparing free heme described by PAUL which had been successfully applied to mammalian cyt. *c*[23] was first tried for this pigment. The method

* In this case, the change of the optical density at 418 mμ (Soret band of reduced cyt. b_4) was observed.

employs silver nitrate to break the thioether bonds between the heme and the protein. Only part of the heme was liberated into the ether–acetic acid layer. Therefore, pyridine hemochromogens of Prep. 4, as well as purified cyt. b_4, type I, and beef heart cyt. *c*, were prepared by alkaline treatment in the presence of pyridine in order to determine the heme structure. The absorption maxima of these three hemochromogens at α-, β-, and Soret bands were identical, indicating a common heme structure for the *c*-type cytochrome.

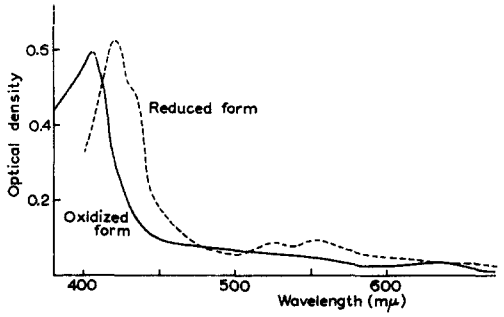


Fig. 2. Absorption spectra of Micrococcus hydroxylamine reductase preparation. Components: hydroxylamine reductase, Prep. 4; phosphate buffer, 0.05 *M*, pH 6.8; total volume, 3.0 ml. Reduced by dithionite.

TABLE IV

ABSORPTION PEAKS OF MICROCOCCUS CYTOCHROMES

Cytochromes	*Absorption peaks (mμ)*	
	Reduced form	*Oxidized form*
Cyt. b_4 type I	554, 548, 521, 418	523, 414
type II	554, 521, 418	523, 414
Hydroxylamine reductase	554, 521, 420	635, 405

Spectrophotometric measurement of HdR activity

As shown in Fig. 3, under the assay conditions of the spectrophotometric method, the optical density of reduced HdR at 420 mμ in Prep. 4 decreased after the addition of hydroxylamine when the reduced spectrum shifted completely to the oxidized one, after which no further decrease in optical density was observed. The presence of Mn ions was again found to stimulate the rate of the reaction.

Reaction of reduced HdR with molecular oxygen

The indication that HdR was a cytochrome led the authors to examine the reaction of HdR with oxygen. It was found that the reduced enzyme reacted with oxygen and that the reaction was again accelerated by Mn ions (Fig. 4). The rate of oxidation of reduced HdR by oxygen is higher than that by hydroxylamine.

There is a linear relationship between the specific activities of HdR measured by the MbH_2 method and of the autoxidizability measured by the spectrophotometric method (Fig. 5), which indicates that HdR can react with two substrates.

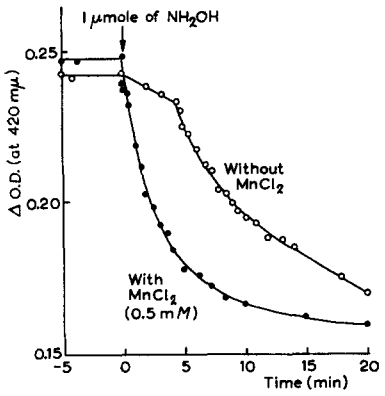


Fig. 3. Oxidation of reduced form of hydroxylamine reductase preparation by NH_2OH and the effect of Mn ions on the oxidation. Standard spectrophotometric assay conditions using Prep. 4.

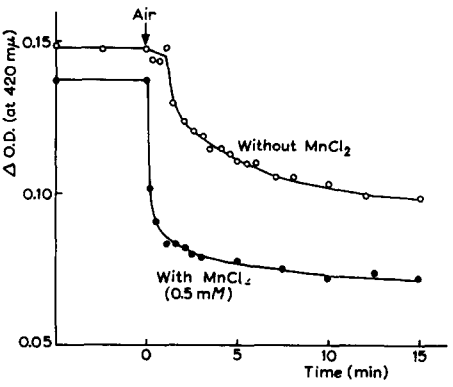


Fig. 4. Oxidation of reduced form of hydroxylamine reductase preparation by oxygen and the effect of Mn ions. Standard spectrophotometric assay conditions using Prep. 4.

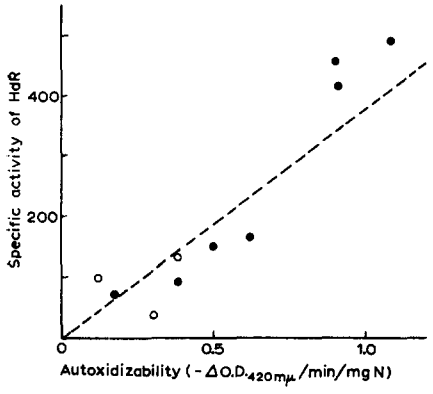


Fig. 5. Relation between hydroxylamine reductase activity and autoxidizability of various preparations: ●, Prep. 4; ○, Prep. 3.

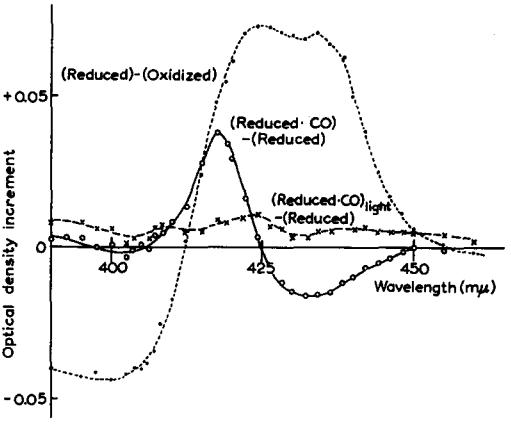


Fig. 6. CO-binding difference spectrum and photodissociation. Standard spectrophotometric assay conditions using Prep. 4. For details see text.

Reaction with carbon monoxide

Using the MbH_2 method, photoreversible inhibition of the HdR activity by CO had been reported previously[3,4]. To confirm this effect of CO on HdR spectrophotometrically, one atm. of CO was introduced into a Thunberg tube-type cuvette which contained reduced HdR.

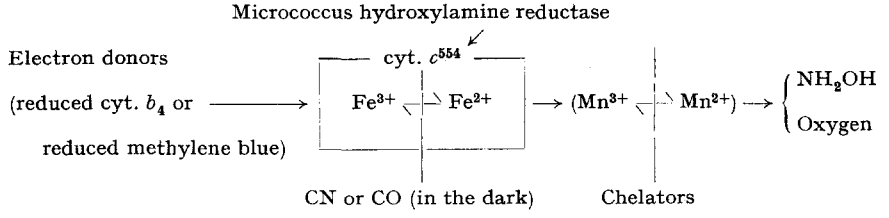
As Fig. 6 shows, there is a marked change in the spectra—disappearance of the shoulder at 434 mμ. When the cuvette, with reduced CO pressure (*ca.* 0.02 atm.) was illuminated by a 100 Watt tungsten lamp at a distance of 30 cm, the CO effect was completely reversed. No photoreversibility was observed with less than 1 atm. of CO. These observations confirm the previous experiments with the MbH_2 method on the inhibitory effects of CO on HdR.

DISCUSSION

The results of metal analyses of HdR preparations containing more than one electrophoretic and ultracentrifugal component, give us considerable information about the active center of HdR. The most striking fact is that only iron was found in the dialyzed Prep. 3. The dissociable nature of Mn was also supported by these experiments. From the inhibitory effects of KCN and CO on HdR[3,4], it has been suggested that iron, as the active center of HdR, undergoes a valency change during hydroxylamine reduction. On the other hand, the presence of dialyzable manganese ions seems to explain the inhibitory effects of various chelating agents[3,4].

As a possible mode of action for the Mn ions, their participation in the formation of an enzyme–substrate complex, probably with a simultaneous valency change, is proposed. The reproducible straight line in LINEWEAVER-BURK's plot obtained with a constant $MnCl_2/NH_2OH$ molar ratio of 1/5 can be also explained by the assumption that the true substrate for the HdR is not free hydroxylamine but a complex of hydroxylamine and Mn ions. Unlike the case of the complex ion $(MgP_2O_7)^{=}$ supposed to be the true substrate for rat erythrocyte pyrophosphatase[24] however, neither evidence nor any indication of the existence of such a complex has been put forward. Similar activation by manganese ions has been observed with Azotobacter HdR; however it has not yet been determined whether the enzyme is a hemoprotein. On the contrary, though the modes of action for the manganese ions during oxygen reduction (Fig. 4) were not studied, the possibility of a valency change as in the case of plant peroxidase[25] seems to be again probable in view of the susceptibility of Mn^{2+} to autoxidation under physiological conditions. Thus, the functioning step of manganese ions seems to be the last one which follows the electron transfer of HdR by heme irons.

The occurrence in the present organism of a soluble, autoxidizable and carbon monoxide-binding cytochrome has been reported[11] and a possible relation between this cytochrome and HdR is suggested. That the cytochrome is actually HdR is proved by the experiments reported here—firstly, the linearity between the HdR activity and autoxidizability (Fig. 5), secondly, the inhibition of HdR activity by CO[3,4] and its effect on HdR spectra (Fig. 6) and finally the spectroscopic change of the absorption band caused by the addition of hydroxylamine (Fig. 3). Therefore from these results HdR can be represented as cyt. c^{554} (halotolerant Micrococcus, CO-binding). The above findings are summarized schematically in the following scheme:



Some heme proteins such as human hemoglobin[26] and an unidentified cytochrome found in mammalian liver[27] have been reported to react with hydroxylamine in a similar manner to that described above. Observations recently reported by ISHIMOTO

et al.[28] and SENEZ *et al.*[29] are essentially interesting in connection with our results. They found that cyt. c_3 produced from *Desulfovibrio desulfuricans* is able to catalyze the reduction of hydroxylamine by MVH. This HdR activity is inhibited by CO; this inhibition was reversed by light and the reduction was remarkably stimulated by Mn ions. The HdR activity of cyt. c_3 could also be confirmed by our MbH_2 method. MINAKAMI *et al.*[30] have also shown that heme-peptides prepared from mammalian cyt. *c* by tryptic digestion possess both HdR activity and CO-binding capacity, although intact cyt. *c* does not. The effect of Mn ions was, however, not observed in this case. These findings seem to suggest that cytochromes of the *c*-type are able to acquire an HdR activity and CO-binding capacity if their structures are modified moderately. It seems, however, quite improbable that the HdR obtained by us is an artifact in view of the mildness of the procedures employed and the consistent amounts recovered in each step of the purification.

In this connection it is noteworthy to mention that cyt. b_4 is completely inert toward hydroxylamine as well as toward oxygen and CO in the reduced form. Recently, *Rhodospirillum rubrum* hemoprotein[31] has been shown to have properties quite similar to that of HdR, although its reduced form lacks a β-band. Recently, these and chromatium cytochromes[32] and CHANCE's "new-CO-binding pigment", *e.g.*, from *Staph. albus*[33] have been tentatively classified by MORTON[34] under the new heading of "cyt. *d*" which is characterized by *c*-heme, autoxidizability and CO-binding capacity. Further extensive studies seem to be still required before those findings can be generalized to give the characteristics of a certain group of hemoproteins.

The presence of CHANCE's "new CO-binding pigment" or "cyt. *o*"[35] which is regarded as a new type of terminal oxidase has been also confirmed in the halotolerant Micrococcus employed in this study[36]. No evidence, however, has been obtained for the possibility that the soluble, autoxidizable and CO-binding cyt. c^{554} which shows HdR activity is a terminal oxidase functioning *in vivo*—on the contrary, almost all of *p*-phenylene diamine oxidizing activity originally present in the lyophilized cells was found in the precipitate after centrifugation (105,000 $\times$ *g* $\times$ 60 min), while the HdR activity was found exclusively in the supernatant. Furthermore, the abundant presence of a CO-binding cytochrome in the precipitate has been confirmed. These observations seem to rule out the possibility that HdR functions as the predominant terminal oxidase in the Micrococcus.

The final purification and the study of the physiological significance of HdR, as well as the biochemical comparison of HdR with an uncharacterized particulate CO-binding cytochrome found in the same Micrococcus are now under way.

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REFERENCES

1 S. TANIGUCHI, A. ASANO, K. IIDA, M. KONO, K. OHMACHI AND F. EGAMI, *International Symposium on Enzyme Chemistry, Tokyo and Kyoto*, 1957, Maruzen Co., Ltd., Tokyo, 1958, p. 238.
2 S. TANIGUCHI, H. MITSUI, J. TOYODA, T. YAMADA AND F. EGAMI, *J. Biochem. (Tokyo)*, 40 (1953) 175.
3 S. TANIGUCHI, H. MITSUI, K. NAKAMURA AND F. EGAMI, *Ann. Acad. Sci. Fennicae, Ser. A, II*, 60 (1955) 200.
4 S. TANIGUCHI, R. SATO AND F. EGAMI, in W. D. MCELROY AND B. GLASS, *Inorganic Nitrogen Metabolism*, The Johns Hopkins Press, Baltimore, U.S.A., 1956, p. 87.
5 M. ZUCKER AND A. NASON, *J. Biol. Chem.*, 213 (1955) 463.
6 A. NASON, R. A. ABRAHAM AND B. C. AVERBACH, *Biochim. Biophys. Acta*, 15 (1954) 160.
7 D. SENCER, H. TAKAHASHI AND A. NASON, *J. Bacteriol.*, 73 (1957) 553.
8 A. ASANO, *J. Biochem. (Tokyo)*, 46 (1959) 781, 1235.
9 M. D. KAMEN, personal communication.
10 S. TANIGUCHI *et al.*, manuscript in preparation.
11 M. KONO, S. TANIGUCHI AND F. EGAMI, *J. Biochem. (Tokyo)*, 44 (1957) 615.
12 D. KEILIN AND E. F. HARTREE, *Proc. Roy. Soc. (London), B*, 124 (1938) 397.
13 H. BOMAN AND L. E. WESTLUND, *Arch. Biochem. Biophys.*, 64 (1956) 217.
14 R. SATO AND F. EGAMI, *Bull. Chem. Soc. Japan*, 22 (1949) 137.
15 F. EGAMI, *Bull. soc. chim. biol. (Paris)*, 37 (1955) 207.
16 T. Z. CSÁKY, *Acta Chem. Scand.*, 2 (1948) 450.
17 E. KAHANE, *Ann. chim. anal. et chim. appl.*, 17 (1935) 175.
18 O. H. LOWRY, N. J. ROSEBROUGH, A. L. FARR AND R. J. RANDALL, *J. Biol. Chem.*, 193 (1951) 265.
19 E. B. SANDEL, *Colorimetric Determination of Traces of Metals*, Interscience Publishers Inc., New York, 1950, p. 537.
20 H. KIYOTA AND T. YAMAMOTO, *Bull. Chem. Soc. Japan*, 76 (1954) 1179.
21 E. B. SANDEL, *Colorimetric Determination of Traces of Metals*, Interscience Publishers Inc., New York, 1950, p. 662.
22 R. SATO, manuscript in preparation.
23 K.-G. PAUL, *Acta Chem. Scand.*, 4 (1950) 239.
24 L. BLOCH-FRANKENTHAL, *Biochem. J.*, 57 (1954) 87.
25 R. H. KENTEN AND P. J. G. MANN, *Biochem. J.*, 45 (1949) 255.
26 J. S. COLTER AND J. H. QUASTEL, *Arch. Biochem.*, 27 (1950) 368.
27 I. RAW, *Experientia*, 12 (1956) 348.
28 M. ISHIMOTO, T. YAGI AND M. SHIRAKI, *J. Biochem. (Tokyo)*, 44 (1957) 707.
29 J. C. SENEZ AND F. PICHINOTY, *Biochim. Biophys. Acta*, 28 (1958) 355.
30 S. MINAKAMI, K. TITANI, H. ISHIKURA AND K. TAKAHASHI, *International Symposium on Enzyme Chemistry*, Tokyo and Kyoto, 1957, Maruzen Co., Ltd., Tokyo, 1958, p. 211.
31 R. G. BARTSCH AND M. D. KAMEN, *J. Biol. Chem.*, 230 (1958) 41.
32 R. G. BARTSCH AND M. D. KAMEN, *J. Biol. Chem.*, 235 (1960) 825.
33 B. CHANCE, *J. Biol. Chem.*, 202 (1953) 383.
34 R. K. MORTON, *Rev. Pure and Appl. Chem. (Australia)*, 8 (1958) 161.
35 L. N. CASTOR AND B. CHANCE, *J. Biol. Chem.*, 234 (1959) 1587.
36 B. CHANCE, *Discussions Faraday Soc.*, No. 20 (1955) 205.