132. The Enzymatic Degradation of Heme Proteins into Bile Pigments. XI (1)

The Enzymatic Degradation of Hemoglobin-haptoglobin Complex into a Precursor of Biliverdin. I

By Osamu NAKAJIMA

First Department of Internal Medicine, Faculty of Medicine,
Kyushu University, Fukuoka

(Comm. by N. ONODERA, M.J.A., Oct. 12, 1962)

In the previous communications,¹⁾ heme α -methenyl oxygenase, a new enzyme which capable of transforming pyridine-hemichromogen into a direct precursor of biliverdin in the presence of TPNH and ferrous iron was isolated from animal liver and characterized. In reference to the substrate specificity of this enzyme, it was found, that the hemoglobin haptoglobin complex rather than free hemoglobin is the true substrate in this reaction.²⁾ Furthermore, one of the biological role of haptoglobin, a hemoglobin-binding plasma protein, is established in the enzymatic degradation of hemoglobin *in vivo*.

The present communication describes the enzymatic degradation of hemoglobin-haptoglobin complex into a precursor of biliverdin by guinea-pig liver enzyme.

Experimental Procedures. *Preparation of Tissue Homogenate.* The procedure employed was the same as described in the previous communication.¹⁾

Preparation of the Intracellular Particulate Fraction. The method used was essentially the same as described by Schneider,³⁾ and a 0.25M sucrose solution was used as the homogenization medium.

Preparation of the Crude Enzyme Preparation. Guinea-pig livers were homogenized in four volumes of 0.1M sodium phosphate buffer of pH 7.4 with a Potter-Elvehjem homogenizer. After a preliminary centrifugation at $10,000 \times g$ for 15 minutes, the supernatant solution was further centrifuged at $105,000 \times g$ for 60 minutes. The particulate-free supernatant thus obtained was used as the crude enzyme preparation.

Preparation of Substrate. Destromatized hemolysate prepared from human blood was used as a hemoglobin solution in these experiments, and the concentration was estimated in a Beckman spectrophotometer Model DU using Drabkin's extinction coefficient for oxyhemoglobin.⁴⁾

Preparation of Hemoglobin-haptoglobin Complex. A solution of hemoglobin-haptoglobin complex was prepared by adding hemoglobin

to a partially purified haptoglobin solution from human serum (Hp₂₋₁) in an amount just sufficient to saturate the hemoglobin. The correct amount was determined by preparing a series of a mixtures of increasing amounts of hemoglobin with a constant volume of partially purified haptoglobin solution. The sample in which free hemoglobin appeared in the plasma was determined by paper electrophoresis according to Owen,⁵⁾ using *o*-dianisidine staining.

Partial Purification of Haptoglobin. Haptoglobin or a hemoglobin-binding plasma protein was partially purified from human serum (Hp₂₋₁) by means of ammonium sulfate fractionation. Most of this protein was precipitated between 0.33 to 0.50 saturation.

Assay Method. In routine experiments, the reaction mixture consists of 1.0 ml of the crude enzyme preparation (60 to 80 mg protein per ml), 0.2 ml of hemoglobin solution (1,700 mg per dl), partially purified haptoglobin solution just sufficient to saturate the hemoglobin, and 0.1 M phosphate buffer, pH 7.4, at a final volume of 2.0 ml. The activity was assayed at 37° and pH 7.4 aerobically by the conventional Warburg's technique. A control experiment with boiled enzyme preparation was performed at the same time. One unit of enzyme was defined as that amount which caused an oxygen uptake of 1.0 μ l per 30 minutes of incubation. Specific activity was defined as the number of enzyme units per mg of protein. Protein was measured by the Kjeldahl method or the method of Lowry⁶⁾ with crystalline bovine albumin (Armour and Company, Chicago) as a standard solution.

Results and Discussions. *Conversion of Hemoglobin-haptoglobin into a Precursor of Biliverdin by the Liver Homogenate.* It was demonstrated that the liver homogenate catalyzed the conversion of hemoglobin-haptoglobin to a precursor of biliverdin with the consumption of oxygen. The activity was lost by treatment of the homogenate at 80° for 5 minutes at pH 7.4. The activity was decreased by dialysis; however, the activity was found to be recovered on the additions of boiled liver extracts or dialysate to the dialyzed homogenate. It was failed to detect any oxygen consumptions upon omission of one or more of the constituents of the reaction mixture

Table I. Conversion of hemoglobin-haptoglobin by liver homogenate

System	Activity ($-O_2\mu$ l/60 minutes)
Complete system	44.8
Without hemoglobin	14.3
Without haptoglobin	4.5
Without homogenate and hemoglobin	12.6
Without homogenate and haptoglobin	0.6
Without hemoglobin and haptoglobin	7.4

(Table I). These facts definitely indicate the enzymatic nature of this reaction.

The Distribution of the Enzyme Activity in Different Animal Tissues. The specific activities of the enzyme in the various tissues are presented in Table II. It is clearly seen that the activity was high in liver and kidney, whereas very low in spleen.

Intracellular Distribution of the Enzyme Activity. Table III shows that most of activity is distributed in supernatant fraction, while the large granules as well as the small granules are almost without activity. The sucrose solution seemed not to exhibit the direct inhibitory effect on the enzymatic reaction, however, the enzyme was inactivated in a medium of a 0.25 M sucrose solution without pH adjustment. Therefore, it was necessary to adjust the pH of the sucrose solution to 7.4 before the homogenization procedure.

Several Properties of the Enzyme When the Supernatant Solution was used as an Enzyme Preparation. The activity was proportional

Table II. The distribution of the enzyme activity in different animal tissues

Tissue	Total activity (units)	Specific activity (units/mg protein)
Guinea-pig liver	142.7	0.53
„ kidney	32.9	0.13
„ lung	2.3	0.01
„ spleen	22.8	0.09

The reaction mixture consists of 1.0 ml of the tissue homogenate indicated in the table (60 to 80 mg protein/ml), 0.2 ml of hemoglobin solution (1,700 mg/dl), partially purified haptoglobin solution just sufficient to saturate the hemoglobin, and 0.1 M phosphate buffer, pH 7.4, at a final volume of 2.0 ml. The activity was assayed as described in the text.

Table III. Intracellular distribution of the enzyme activity

Fractions	Vol (ml)	Activity (units/ml)	Protein (mg/ml)	Specific activity (units/mg protein)	Total activity (units)
Homogenate	12.0	130.83	276.9	0.509	1569.9
Nucl. frac	12.5	19.53	171.1	0.113	244.1
Large gr	25.0	2.03	51.6	0.039	50.8
Small gr	12.5	2.45	163.5	0.015	30.6
Supern.	51.2	17.52	47.3	0.370	897.0

The reaction mixture consists of 1.0 ml of the fraction mentioned in the table (60 to 80 mg protein/ml), 0.2 ml of hemoglobin solution (1,700 mg/dl), partially purified haptoglobin solution just sufficient to saturate the hemoglobin, and 0.1 M phosphate buffer, pH 7.4, at a final volume of 2.0 ml. The activity was assayed as described in the text.

Fig. 1. The enzyme activity as a function of the enzyme concentrations. The reaction mixture consists of 0.2 ml of hemoglobin solution (1,700 mg/dl), partially purified haptoglobin solution just sufficient to saturate the hemoglobin, 0.1 M phosphate buffer, pH 7.4, and enzyme solution indicated above as a final volume of 2.0 ml. The activity was assayed as described in the text.

Fig. 2. The enzyme activity as a function of substrate concentration. The reaction mixture consists of 1.0 ml of the enzyme solution (80 mg protein/ml), 0.1 M phosphate buffer, pH 7.4, hemoglobin solution indicated in the table, and partially purified haptoglobin solution just sufficient to saturate the hemoglobin.

Fig. 3. Time course of the enzyme reaction.

○—○ Methemoglobin-haptoglobin₂₋₁ ●—● Oxyhemoglobin-haptoglobin₂₋₁.

Fig. 4. pH-activity curve of the reaction.

●—● 0.2 M phosphate buffer ○—○ 0.2 M tris buffer.

Fig. 5. Substrate specificity from the viewpoint of haptoglobin-types.

○—○ Haptoglobin₂₋₂-methemoglobin ●—● Haptoglobin₂₋₁-methemoglobin
□—□ Haptoglobin₁₋₁-methemoglobin.

to enzyme concentrations below 120 mg of protein (Fig. 1). The K_m value for substrate was estimated to be approximately 3.39×10^{-4} M from the Lineweaver-Burk plot⁷⁾ (Fig. 2). Oxygen uptakes were linear within 60 minutes to the time of the incubation after 5 minutes of lag phase in oxyhemoglobin and without any lag phase in methemoglobin as a substrates (Fig. 3). The enzymatic activities were determined at different pH, phosphate and tris buffer used. Similar activity, however, was observed between them. The results are presented in Fig. 4. It will be clearly seen that the optimum pH for the activity lies at 7.5.

Substrate Specificity. (A) The site of hemoglobin derivatives. A study on the degradation of hemoglobin-haptoglobin from the viewpoint of hemoglobin derivatives has disclosed that oxygen uptake is greater in carboxyhemoglobin-haptoglobin₂₋₁ and methemoglobin-haptoglobin₂₋₁ than in oxyhemoglobin-haptoglobin₂₋₁ (Table IV).

Table IV. The substrate specificity from the viewpoint of hemoglobin derivatives

Substrates	Activity (units)
Oxyhemoglobin-haptoglobin ₂₋₁	17.8
Methemoglobin-haptoglobin ₂₋₁	24.8
Carboxyhemoglobin-haptoglobin ₂₋₁	25.6

The reaction mixture consists of 1.0 ml enzyme solution (60 mg protein/ml), 0.2 ml hemoglobin solution, partially purified haptoglobin (Hp₂₋₁) just sufficient to saturate the hemoglobin and 0.1 M phosphate buffer, pH 7.4, at a final volume of 2.0 ml. Hemoglobin derivatives are estimated by Beckman spectrophotometer Model DK₂. The activity assayed as described in the text.

(B) The site of haptoglobin types. Fig. 5 shows that the initial velocity of the degradation is greater in methemoglobin-haptoglobin₂₋₂ than in methemoglobin-haptoglobin₂₋₁ or methemoglobin-haptoglobin₁₋₁ (Fig. 5). The mechanism of these differences and the biological significance are currently under study.

Summary. (1) The conversion of hemoglobin-haptoglobin into a precursor of biliverdin by animal liver homogenate is identified as an enzymatic reaction.

(2) The observation of the enzyme activity in different animal tissues has revealed that the activity is high in liver and kidney, and extremely low in spleen.

(3) It has been definitely shown by the use of the cell-fractionation technique that the activity is distributed in the supernatant fraction.

(4) The enzyme kinetics was described by the use of supernatant

fraction of the liver homogenate as an enzyme preparation.

(5) In reference to the substrate specificity of this enzyme, it was shown that the initial velocity of the degradation was greater in methemoglobin-haptoglobin₂₋₂ than in methemoglobin-haptoglobin₂₋₁ or methemoglobin-haptoglobin₁₋₁. From the viewpoint of hemoglobin derivatives, it has disclosed that the oxygen uptake is greater in carboxyhemoglobin-haptoglobin₂₋₁ or in methemoglobin-haptoglobin₂₋₁ than in oxyhemoglobin-haptoglobin₂₋₁.

Acknowledgement. The author is greatly indebted to Professor K. Yamaoka, First Department of Internal Medicine, Kyushu University and to Associate Professor H. Nakajima, Department of Clinical Genetics, Tokyo Medical and Dental University, and to Dr. M. Yamaguchi, for their valuable advices and encouragement during this work.

This study was partially supported by U.S. Public Health Service Grant Fund (RG 9470) from the National Institute of Health and by Scientific Research Fund of Ministry of Education of Japan.

References

- 1) Nakajima, H.: Proc. Japan Acad., **34**, 712 (1958).
- 2) Yamaguchi, N., Nakajima, H., and Yamaoka, K.: Ibid., **37**, 584 (1961).
- 3) Schneider, W. C.: J. Biol. Chem., **176**, 259 (1948).
- 4) Drabkin, D. L.: J. Biol. Chem., **185**, 231 (1950).
- 5) Owen, J. A., Silberman, H. J., and Got, C.: Nature, **182**, 231 (1950).
- 6) Lowry, O. H., Rosebrough, N. J., Farr, A. L., and Randall, R. J.: J. Biol. Chem., **193**, 265 (1951).
- 7) Lineweaver, H., and Burk, D. J.: J. Amer. Chem. Soc., **56**, 658 (1934).