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Catalytic Properties of Cytochrome c Heme Peptides

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Several kinds of heme peptides of cytochrome c were prepared and examined on their two catalytic properties, cytochrome c oxidative effect and peroxidative effect. The unrigid coordination at the sixth ligand place of heme iron in heme peptide was proved to be responsible for the two effects. The characteristic roles of peptide moiety of heme peptide in the effects were also discussed.

It is an interesting attempt to search a peptide fragment possessing characteristic biological properties in a partial amino acid sequence of a protein whose primary structure has already been determined. We have investigated the several biological and chemical properties of the peptide fragments obtained by the enzymatic and chemical cleavages of cytochrome c.[2,3]

Cytochrome c, a member of the mitochondrial electron transport chain system, is a heme protein readily obtained pure from various sources, and amino acid sequences of a number of cytochrome c have been elucidated successively in the last few years. In contrast with many other heme proteins, *e.g.* cytochrome a, hemoglobin, and peroxidase, the heme and peptide component of cytochrome c are tightly coupled *via* thioether linkages, consequently, the cleavages of peptide bonds permit the isolations of small heme combining fragments (heme peptides). In this report we present the results of the studies on some biological properties of such heme peptides.

As pointed out first by Tuppy and Paleus,[4] heme peptides show marked peroxidative activity. In addition, we found characteristic cytochrome c oxidative activity.[5] We examined the two activities in detail from the point of view of the relations between the activities and the chemical structures of heme peptides.

Experimental

Materials

Cytochrome c——The present studies were carried out mainly with cytochrome c from *Saccharomyces oviformis* M_2,[6] but some experiments were also made with the material from equine hearts. All such cytochromes c were obtained chromatographically pure in our pharmaceutical industries.

Trypsin, Chymotrypsin and Pepsin——These proteolytic enzymes were obtained from Shigma Chemical Co.

Nagarse——This was obtained from Nagase Sangyo Co., Ltd. All other chemicals were reagent grade.

Preparations of Heme Peptides——Several kinds of heme peptides were prepared by enzymatic hydrolysis of the *Saccharomyces* cytochrome c. Those from equine heart cytochrome c were also prepared by exactly the same procedure. The method of preparations for the *Saccharomyces* cytochrome c heme peptides are described below. The amino acid sequences of each heme peptide are illustrated in Fig. 1 and the amino acid analysis data of the present preparations are shown in Table I.

1) Location: *1-2-58, Hiromachi, Shinagawa-ku, Tokyo.*
2) Y. Baba, H. Mizushima, A. Ito and H. Watanabe, *Biochem. Biophys. Res. Commun.*, **26**, 505 (1967).
3) H. Watanabe, M. Murata, K. Kitamura, Y. Baba and H. Mizushima, "7th International Congress of Biochemistry," H-14, Tokyo, 1967.
4) S. Paleus, A. Ehrenberg and H. Tuppy, *Acta Chem. Scand.*, **9**, 365 (1965).
5) Y. Baba, H. Mizushima and H. Watanabe, *Chem. Pharm. Bull.* (Tokyo), **16**, 763 (1968).
6) K. Narita, *Biochem. Biophys. Acta*, **77**, 688 (1963).

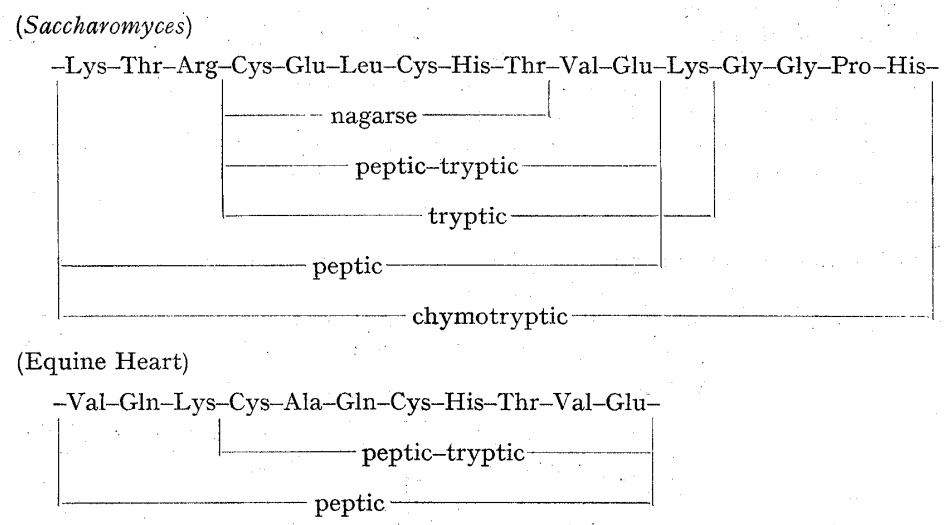

Fig. 1. Amino Acid Sequences of Cytochrome c Heme Peptides

Table I. Amino Acid Ratios of the Heme Peptides prepared in the Present Study

Heme Peptide	Lys	His	Arg	Thr	Glu	Pro	Gly	Ala	Val	Cys	Leu
Sacchromyces nagarse		0.79		0.79	2.00					—	0.84
Sacch. tryptic	0.75	0.84		0.97	2.00				0.86	—	1.17
Sacch. peptic	0.77	0.86	0.77	1.86	2.00				1.00	—	1.04
Sacch. chymotryptic	1.64	1.79	0.91	1.90	2.00	1.08	1.91		1.02	—	1.05
Equine heart peptic	0.98	0.89		0.87	3.00			0.93	0.96	—	
Equine heart peptic–tryptic		0.92		0.89	2.00			0.97	0.98	—	

Peptic Heme Peptide: It was prepared following the method of Tuppy, *et al.*[4] for equine heart cytochrome c peptic heme peptide.

Tryptic Heme Peptide: The solution of cytochrome c (500 mg) and trypsin (25 mg) in 1 N ammonium bicarbonate (100 ml) was allowed to stand at 37° for 15 hours. The reaction mixture was liophilized and the resulting powder was redissolved in water (100 ml) followed by the addition of 30% trichloroacetic acid solution (50 ml). The dark red precipitates were collected by centrifugation, washed with a small amount of water, dissolved in 0.01 M ammonia and dialyzed several times against 0.1 M phthalate buffer pH 5.0 and subsequently against water. By the liophilization of the resulting solution, red fluffly materials were obtained (The procedure up to this point was also applied to the preparations of nagarse and chymotryptic heme peptides described below). The powder thus obtained were not homogeneous as the result of amino acid analysis and subsequent purifications were performed by a chromatography on DEAE Sephadex A 25 column (2.5×40 cm) by eluting with a linear gradient system of volatile buffers from 1% pyridine–0.1% acetic acid to 1% pyridine–0.6% acetic acid (1 liter each). The colored fraction was collected and concentrated *in vacuo* below 50° to complete dryness, and the residue was dissolved in a small amount of 0.01 M ammonia and liophilized. Somewhat hygroscopic dark red powder was obtained. Yield 25 mg.

Nagarse Heme Peptide: Cytochrome c (500 mg) was hydrolyzed by nagarse (20 mg) in 1 N ammonium bicarbonate (100 ml) at 37° for 15 hours. Crude heme peptide was isolated analogously to the preparation of tryptic heme peptide. Chromatographical purification was performed by a DEAE Sephadex A 25 column (2.5×40 cm). The elution was carried out by a linear gradient system from 1% pyridine–0.1% acetic acid to 1% pyridine–0.5% acetic acid (1 liter each). The latter of the two colored bands was collected and treated as the case of tryptic heme peptide. Dark red powder was obtained. Yield 29 mg.

Chymotryptic Heme Peptide: Cytochrome c (500 mg) was hydrolyzed by chymotrypsin (25 mg) in 1 N ammonium bicarbonate (100 ml) at 37° for 15 hours, and crude heme peptide was isolated analogously to the preparation of tryptic heme peptide. Chromatographical purification was carried out on a column of IRC 50 (3×40 cm) eluted by a linear gradient system from 0.1 N to 0.2 N ammonium phosphate buffers pH 7.0 (1 liter each). The main and last of the well separated three colored bands was collected and readsorbed to IRC 50 (H^+ type) followed by reelution with a small amount of 1 N ammonium phosphate buffer pH 7.0 in order to reduce the volume of the effluent. After dialysis against several changes of water and liophilization, a red powder was obtained. Yield 63 mg.

Peptic–tryptic Heme Peptide: Peptic heme peptide (30 mg) was hydrolyzed by trypsin (3 mg) in 1 N ammonium bicarbonate (20 ml) at 37° for 15 hours. The reaction mixture was evaporated to dryness *in vacuo*. The residue was dissolved in a small amount of the upper layer from a freshly prepared mixture of *n*-buthanol, glacial acetic acid and water (4:1:5) and passed through the column of hyflosupercel (3×7 cm) suspended in the same solvent mixture in order to eliminate trypsin. The dark brown effluent was collected and evaporated to dryness *in vacuo* and the residue was redissolved in as little 0.01 N ammonia as possible, dialyzed several times against water. Liophilization afforded dark red powder. Yield 16 mg.

Chemical Modifications of Heme Peptide——The amino group of the peptic heme peptide from the *Saccharomyces* cytochrome c were chemically modified in three ways.

Acetylation: Peptic heme peptide (100 mg) was mixed with acetic anhydride (100 mg) in 1 N K_2HPO_4 (20 ml) and the resulting mixture was stirred for an hour with ice cooling. The modified heme peptide solution was dialyzed against several changes of water and liophilized, then acetylated heme peptide was obtained. Ninhydrin color reaction of the material was negative.

Succinylation: To the solution of the heme peptide (100 mg) in 1 N K_2HPO_4 (20 ml) finely powdered succinic anhydride (500 mg) was added in several portions with vigorous stirring under ice cooling over a two-hour period. The pH of the reaction mixture was kept at 8.0 by the automatic addition of 1 N sodium hydroxide by a pH–stat. The resulting solution was dialyzed against several changes of water. (During the dialysis a considerable amount of the modified heme peptide was lost). The ninhydrin color reaction for the succinylated peptide thus obtained was completely negative.

Guanidylation: The heme peptide (100 mg) was treated with 1-guanidino-3,5-dimethylpyrazol[7] (200 mg) in 1 N K_2HPO_4 (20 ml) at 5° for a week. The resulting solution was dialyzed several times against water and liophilized. Amino acid analysis showed that lysine was disappeared, consequently, the amino groups were guanidylated exhaustively.

Methods

Spectral Data——Spectra were recorded with Cary Model 14. The concentrations of heme peptides for the spectral measurement were adjusted to 3×10^{-6}M.

Determination of the Concentrations of Heme Peptide Solutions——Concentrations of heme peptide and modified heme peptide solutions were determined by the amino acid analysis. A portion of a heme peptide solution was combined with the equal volume of conc. hydrochloric acid and the mixture was heated at 110° for 24 hours in evacuated sealed tube. The hydrolysate was analyzed as usual by amino acid analyzer (Hitachi KLA–2). The concentrations of heme peptide solution were calculated according to the value for leucine or alanine. The values for the amino acid analysis were corrected with norleucine, a internal standard.

Preparation of Ferrocytochrome c——Cytochrome c dissolved in a small amount of appropriate buffer, was treated with sodium dithionite and gel filtrated on Sephadex G 25 column according to Yonetani.[8] The concentration of ferrocytochrome c were estimated by the difference in absorbancies at 550 mμ (Δ550) between the oxidized and reduced states. The molal Δ_{550} value for the *Saccharomyces* cytochrome c was 22.0 mM^{-1} cm^{-1}.[9]

Assay of the Cytochrome c Oxidative Activity——The activity was estimated spectrophotometrically by the decrease in absorbancy of ferrocytochrome c at 550 mμ. In general, to a solution of ferrocytochrome c in 0.1 M phosphate buffer pH 7.0 (2.9 ml), heme peptide dissolved in the same buffer (0.1 ml) was added, then the absorption at 550 mμ was recorded. The final concentrations of ferrocytochrome c and heme peptide were adjusted to 50 μM and 5μM each. Assays were carried out in 1 cm spectrophotometer cells, and the total volume of the reaction mixture was adjusted to 3 ml.

One problem was how to avoid the overlapping of the absorptions of ferrocytochrome c and heme peptide at 550 mμ. Generally, reduced heme peptides are immediately oxidized and cannot exist in the presence of oxygen as discussed later. However, in alkaline media containing a large excess of reducing reagents, *e. g.* sodium dithionite, such peptides remain reduced and show a sharp absorption maxima at 550 mμ. To obviate such complications the experiments were carried out at pH below 7.0 and the molar ratios of heme peptide to ferrocytochrome c were kept as low as possible.

Assay of Peroxidative Activity——Peroxidative activity was measured according to the method[10] for the assay of horseraddish peroxidase with slight modifications. Freshly distilled guaiacol was used as a donor. For the determinations of K_1, to the solution of a heme peptide (10^{-6}M) and guaiacol (1.3×10^{-2}M) in 0.01M phosphate buffer pH 8.0,hydrogen peroxide (3.3×10^{-5}M) was added at 20°, and the increase in absorbancy at 470 mμ was checked. The values of K_4 was also estimated by changing the concentrations

7) A.F.S.A. Habeeb, *Canadian J. Biochem. Physiol.*, **38**, 493 (1960).
8) T. Yonetani, *Biochemical Preparations*, **11**, 14 (1966).
9) M. Shirasaka, private communication.
10) "Methods in Enzymology," 2, Academic Press Inc., N.Y., 1955, p. 770. In the method, k_1 shows the velocity constant for the formation of the enzyme substrate complex and k_4 shows the velocity constant for the reaction of the complex with the hydrogen donor complex.

of guaiacol and hydrogen peroxide to 3.3×10^{-4}M and 1.3×10^{-4}M each. The assay was also carried out in spectrophotometer cell. All concentrations are given as final.

Results and Discussions

In Table II the cytochrome c oxidative effects of various heme peptides are shown. The time course study of the effect (Fig. 2) indicated that heme peptides are not merely simple electron acceptors of cytochrome c as ferricyanide but they have catalytic activities, because they oxidize multiple times over equimolecular amount of cytochrome c. As another catalytic property of heme peptides, peroxidative effect was also examined with nine kinds of heme peptides (Table III).

It is well known[11] that with the degradation of peptide bonds in cytochrome c, an originally unobserved peroxidative activity appears with the decrease of its essential oxido-reductive activity. The structural changes around

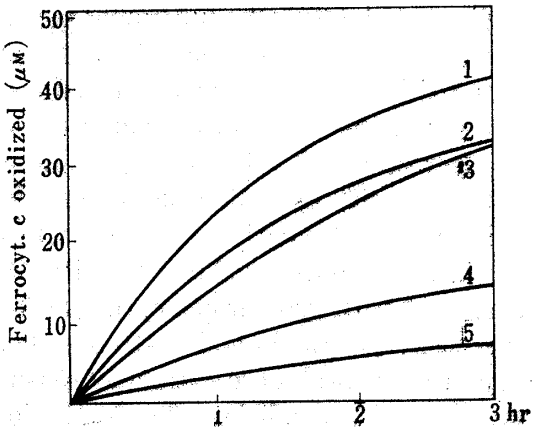

Fig. 2. Time Course Study of the Oxidations of Ferrocytochrome c by Heme Peptides

(1) succinyl peptic, (2) nagarse, (3) peptic-tryptic, (4) peptic, (5) chymotryptic heme peptide (*Saccharomyces*)

Table II. Initial Rates of the Oxidations of Ferrocytochrome c by Various Heme Peptides

Heme peptide (*Saccharomyces*)	Initial rate $\left(\frac{\mu M \text{ ferrocyt. c oxidized}}{\text{sec}}\times10^{2}\right)$	Net charge[a]	Soret maximum (mμ)
Succinyl peptic	1.39	−5	398
Acetyl peptic	0.78	−3	398
Nagarse	0.75	−3	398
Peptic–tryptic	0.60	−4	398
Tryptic	0.30	−3	406
Guanidyl peptic	0.28	−2	404
Peptic	0.24	−2	406
Chymotryptic	0.07	−1	406

a) Net charges were calculated disregarding histidyl residue, and the two carboxyl groups of heme were taken into account.

Table III. Peroxidative Activity of Various Heme Peptides

Heme peptide	$k_1\times10^{-4}$	$k_4\times10^{-4}$	Soret maximum (mμ)
Saccharomyces peptic–tryptic	1.63	3.32	398
Sacch. nagarse	1.50	3.37	398
Equine heart peptic–tryptic	1.50	2.85	398
Sacch. succinyl peptic	1.39	2.81	398
Sacch. acetyl peptic	1.21	2.54	398
Sacch. guanidyl peptic	0.99	1.97	404
Equine heart peptic	0.93	2.02	403
Sacch. peptic	0.57	1.55	406

11) S. Minakami, K. Titani, H. Ishikura and K. Takahashi, "Proceedings of the International Symposium on Enzyme Chemistry, Tokyo and Kyoto," 1957, p. 211.

the heme of cytochrome c by the cleavages of peptide bonds might be responsible for such a change. The properties characteristic of heme peptides must be due to such differences in chemical structure with cytochrome c.

In cytochrome c, four of the six coordination positions of porphyrin iron are occupied by the four pyrrol nitrogens in porphyrin, and the remaining two positions are believed to be coordinated rigidly with functional groups in the protein moiety.[12] This complete and firm coordination by the six ligands give cytochrome c its peculiar oxido–reductive nature and its insensitiveness to oxygen and various externally added ligands. In heme peptide, on the other hand, the states of coordinations are significantly altered in that the sixth position is no longer occupied. The fifth position of heme iron of heme peptide has been shown to be conjugated with the imidazole base of the histidyl residue next to the C-terminal side cystein residue intramolecularly just as cytochrome c,[13] while the sixth position is loosely occupied by water or intermolecularly by nitrogenous group from another heme peptide molecule.[14] In contrast with cytochrome c, the weak ligands at the sixth position of heme peptide are easily replaced by externally added stronger ligands, *e.g.* cyanide, imidazole and pyridine, and especially, heme peptides are extremely sensitive to oxygen and incapable of existing in the ferrous state in aerobic conditions due to the interaction with oxygen at the sixth position. These changes are the basis of various properties of heme peptides including the effects investigated in the present study.

Table IV. Inhibitions of the Cytochrome c Oxidative Effect of Heme Peptides[a]

Without oxygen		100% inhibition
Azide	(10^{-2} M)	39%
Cyanide	(10^{-2} M)	100%
	(10^{-3} M)	95%
	(10^{-4} M)	72%

a) The reactions were carried out by *Saccharomyces* tryptic heme peptide.

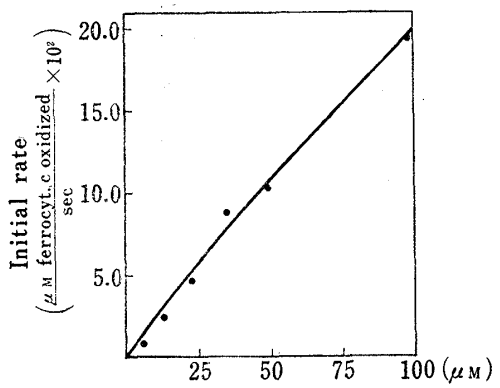

Fig. 3. Proportionality between the Cytochrome c Oxidative Effect and the Amount of Heme Peptide

The reactions were carried out by *Saccharomyces* succinyl peptic heme peptide.

In cytochrome c oxidative effect, cyanide and azide ions, which are the strong ligands with heme iron as shown in the case of hemoglobin and cytochrome oxidase, were proved to be strong inhibitors (Table IV). In addition, the oxidative effect was completely blocked under the anaerobic conditions (Table IV). These results demonstrate that the oxidation is dependent on the interaction between oxygen and heme iron of heme peptides. Probably, an electron is transferred from ferrocytochrome c to heme iron in heme peptide by way of unknown route followed by the immediate migration to oxygen in the reaction medium. Consequently, heme peptides are capable of oxidizing ferrocytochrome c in a catalytic manner (Fig. 2). In Fig. 3 the initial rates with different heme peptide–ferrocytochrome c ratios are plotted. A distinct proportionality was observed.

12) According to R.E. Dickerson, *et al.* (*J. Biol. Chem.*, **242**, 3015, (1967)) one of the iron ligands can be identified as the imidazole side chain of the histidyl residue adjacent to a cysteine linked with heme, and the other is probably located in carboxyl terminal half of the polypeptide chain of cytochrome c.

13) A. Ehrenberg and H. Theorell, *Nature*, **176**, 158 (1955).

14) H.A. Harbury and P.A. Loach, *Proc. Natl. Acad. Sci. U.S.*, **45**, 1344 (1959).

On the other hand the peroxidative effect also decreased with the addition of nitrogenous ligands such as imidazole and pyridine[15] (Table V). These ligands coordinate with the sixth position of heme peptide as demonstrated by the spectral changes (Table V). According to Harbury and Loach[14,16] heme peptide without coordination of a nitrogenous ligand at the sixth position shows the absorption maximum of Soret region at 398 mμ in neutral media, while the coordination of such ligands shifts the maximum to higher wave length. Imidazole and pyridine which alter the Soret maxima to 405 mμ and 402 mμ respectively, weaken the peroxidative activity considerably (Table V). As expected, 2-methylimidazole, 2-methylpyridine, benzimidazole and quinoline which cannot coordinate because of the steric hindrance to the nitrogen functions had no influences on the activity (Table V), but 3- and 4-methylpyridine, whose methyl group does not sterically interfer with the coordination, showed inhibitions similar to pyridine.

Table V. The Peroxidative Activity of Heme Peptides in the Presence of Nitrogenous Ligand externally Added[a]

Nitrogenous ligand (10^{-3} M)	$k_1 \times 10^{-4}$	$k_4 \times 10^{-4}$	Soret maximum (mμ)
None (control)	1.50	3.37	398
Imidazole	0.02	0.58	405.5
2-Methyl imidazole	1.47	3.40	399
Benzimidazol	1.54	3.30	398
Pyridine	1.18	2.93	402
2-Methyl pyridine	1.61	3.59	398
3-Methyl pyridine	1.20	3.02	402
4-Methyl pyridine	0.93	2.63	402.5
Quinoline	1.45	3.15	399

a) The reactions were carried out by *Saccharomyces* nagarse heme peptide.

Thus, the lack of rigid coordination with the sixth ligand have been proved to be indispensable to the effects discussed in this report.

Table II and Table III indicate that both activities vary from heme peptide to heme peptide. The variations can be attributed to the peptide moiety of the respective heme peptides. In such peptides the heme and peptide chain are connected *via* the thioether linkages (Fig. 1), and the histidyl residue is the fifth ligand to heme iron as discussed above. Matters are somewhat complicated as regards sixth lignd. As the spectrophotometric results indicated (Table II and Table III) we can find weak intermolecular coordination of a nitrogenous group such as N-terminal amino group, ε-amino group of a lysine residue or guanidyl group of an arginine residue, when such groups are present in a heme peptide. However, N-terminal amino group of heme peptide, beginning with the N-terminal side cysteine residue connected with heme, are excluded on account of the probable steric hindrance to intermolecular coordination by the bulky heme moiety. For example, in peptic, tryptic, and chymotryptic heme peptides which contain lysine residue, intermolecular coordination were observed as demonstrated by Soret maxima higher than 398 mμ. In nagarse, peptic–tryptic, acetyl peptic and succinyl peptic heme peptides, which lack nitrogenous group for intermolecular coordination, the Soret maxima appeared at 398 mμ (Table II and Table III). The upper parts of Table II and Table III list the heme peptides without intermolecular coordination, while the lower parts those with such a coordination. Marked differences are observed in both the oxidative and peroxidative effects between the two groups. The situation is very similar to the case of externally added ligands.

15) Cyanide and azide ions were inapplicable due to their reactions with hydrogen peroxide.
16) H.A. Harbury and P.A. Loach, *J. Biol. Chem.*, **235**, 3640, 3646 (1960).

About the cytochrome c oxidative effect attention was also directed to the approximately linear relation among each group between the net electronic charges of heme peptide and the activity (Table II). Peptic–tryptic heme peptide is an exception by unknown reason. With increasing negative charges, the activity enhanced. That is to say, the oxidation reaction was certainly stimulated by the electronic affinity of heme peptide to ferrocytochrome c whose isoelectric point was found to be at pH 10—11. An electrostatic interaction like this was observed also in naturally occurred protein–protein interactions. For example, in an mammalian electron transport system, an electron migrates from cytochrome c_1, an acidic heme protein, to cytochrome c, a basic heme protein, and then to cytochrome a, another acidic heme protein, but the blocking of the basic group of cytochrome c, *e.g.* by acetylation, succinylation and trinitrophenylation, interrupted the electron transfer.[17]

These results arouse interest if a heme peptide is considered as an enzyme model: heme plays an essential role in an enzyme action in which the peptide part characterizes its functions in various ways.

17) S. Takemori, K. Wada, K. Ando, M. Hosokawa, I. Sekuzu and K. Okunuki, *J. Biochem.* (Tokyo), **52**, 28(1962).