[Chem. Pharm. Bull. 21(10)2175—2182(1973)] UDC 547.466.1.09

Immunological Cross-Reactivities between Two Yeast Cytochromes c and the Immunological Reactivities of the Peptide Fragments of *Saccharomyces oviformis* M_2 Cytochrome c

Masahiro Murata, Kouichi Kitamura, Yoshihiko Baba, and Hiroshi Watanabe

Central Research Laboratories, Sankyo Co., Ltd.[1]

(Received January 29, 1973)

Cytochromes c from two strains of yeast, *Saccharomyces oviformis* M_2 (M_2-cyt. c) and *Candida krusei*, in their cross-reactivities against their antibodies, were investigated by various methods. The results indicated that cytochromes c from these two yeasts cross-reacted each other with their antibodies, but that horse heart cytochrome c did not cross-react. It was also suggested that the passive cutaneous anaphylaxis inhibition test is preferable to the usual *in vitro* methods in detecting slight difference in the cross-reactivities between closely related antigens.

Another experiments using peptide fragments obtained by enzymatic or chemical cleavage of M_2-cyt. c showed that P-4 and P-7 were found to fix complements, and P-3, P-4, P-5 and P-6 eliminated the inhibitory effect of the antibody against the enzymatic reaction of cytochrome c-cytochrome oxidase.

Immunochemical studies on various types of cytochrome c have been reported from several laboratories, and recently the antigenic determinants of vertebrate cytochromes c were discussed by Margoliash, *et al.*[2] On the other hand, cytochromes c from various yeasts were found considerably different in their amino acid sequences from vertebrate cytochromes c, and their immunochemical properties were also investigated.[3,4]

We employed the cytochromes c from the two strains of yeast, that is, *Saccharomyces oviformis* M_2 (M_2-cyt. c) and *Candida krusei* (CA-cyt. c), for the investigation on the immunological cross-reactivities between substances from homologous species by the passive cutaneous anaphylaxis (PCA) test and by the PCA inhibition test as well as by conventional *in vitro* methods.

Then, the experiments using some relatively long peptide fragments, which were obtained by the enzymatic or chemical cleavages of M_2-cyt. c, were carried out in expecting to obtain further information on the antigenic determinants of yeast cytochrome c. Immunochemical binding affinities of these fragments for *anti*-M_2-cyt. c antibody were compared by the complement fixation (C.F.) test and by the effects against the inhibitory activity of the antibody for the cytochrome c-cytochrome oxidase interaction. Data from these studies contributed to the suggestion of certain regions on the primary structure as the antigenic determinants of yeast cytochrome c.

Experimental

Materials——I) Antigens: M_2-cyt. c and CA-cyt. c were prepared and purified chromatographically according to the modified methods of Okunuki.[5,6] Horse-heart cytochrome c (H.H.-cyt. c) were obtained in high purity by the similar procedure.

1) Location: *Hiro-machi, Shinagawa-ku, Tokyo.*
2) E. Margoliash, A. Nisonoff, and M. Reichlin, *J. Biol. Chem.*, **245**, 931 (1970).
3) S. Watanabe, Y. Okada, and M. Kitagawa, *J. Biochem.* (Tokyo), **62**, 150 (1967).
4) M. Murata, K. Kitamura, Y. Baba, and H. Watanabe, Preprints of the 18th Symposium on Enzyme Chemistry, Sapporo, 1966, p. 350.
5) M. Nozaki, T. Yamanaka, T. Horio, and K. Okunuki, *J. Biochem.* (Tokyo), **44**, 453 (1957).
6) K. Motonaga, E. Misaka, E. Nakajima, S. Ueda, and K. Nakanishi, *J. Biochem.* (Tokyo), **57**, 22 (1965).

Peptide fragments of M_2-cyt. c, seven peptide fragments as shown in Table I were obtained by enzymatic and chemical cleavages of M_2-cyt. c followed by chromatographical separations and purifications.[7] For the hemepeptide, different methods, which were described elsewhere, were employed. Amino acid compositions and purities of the isolated peptides were examined by amino acid analysis and paper chromatography.

Table I. Peptide Fragments derived from M_2-cytochrome c

Fragments	Location in primary structure	Method for cleavage of peptide bonds
P-1	-Lys^{-2}----^{9}Leu-	chymotrypsin
P-2	-Lys^{11}----^{26}His-	chymotrypsin
P-3	-Lys^{27}----^{36}Phe-	chymotrypsin
P-4	-His^{39}----^{54}Lys-	trypsin
P-5	-Asn^{56}----^{73}Lys-	trypsin
P-6	-Ser^{65}----^{79}Lys-	BrCN
P-7	-Ala^{81}----^{103}Glu-	BrCN

II) Antibodies: Male albino rabbits, purchased from a local farm, weighing 2.0—3.0 kg, were immunized by subcutaneous injections of 20 mg of cytochrome c mixed with Freund's complete adjuvant (Difco Co. Detroit) six times during 3 weeks, and boosted intravenously with the same dose of cytochrome c 3 weeks after the last injection. Antisera were collected from the carotial artery of the individual immunized rabbit within 10 days after the booster.

anti-Yeast cytochrome c globulin fraction (G-Fract.) was separated from the above antisera by the precipitation with ammonium sulfate according to the method of Kekwick,[8] and *anti*-yeast cytochrome c-globulin fraction (IgG) was separated by chromatography on DEAE cellulose according to the method of Levy, *et al.*[9]

Assays——Cross-reactivities between yeast and horse cytochromes c against *anti*-yeast cytochromes c antibodies were examined by four methods: the double diffusion test in agar and agarose,[10] the gel-filtration on a Sephadex-G-100 (Pharmacia Co. Uppsala) column, the passive hemagglutination test with tanned sheep erythrocytes coated with antigen,[11] and the C.F. test.[12]

The PCA test in guinea pig was carried out by the modified method of Ovary.[13] The reactions visualized by 5% Evans blue were graded from − to + according to the length and the breadth of coloured region. The PCA inhibition test was carried out as follows. Various concentrations of a contact antigen were coupled with each antibody solution for 2 hours at room temperature before the intradermal injection. The procedure after these treatment were substantially the same as that of the PCA test.

In the C.F. test, the fixation of complement to an antigen–antibody complex was detected by the sheep erythrocyte–hemolysin system. The modified two-dimensional complement fixation test of Kolmer, *et al.*[14] was employed. That is, appropriate dilutions of antigen (M_2-cyt c and peptide fragments) and *anti*-M_2-cyt. c G-Fract. were mixed, and then a specified dose (2 units) of fresh guinea pig serum complements was added and was allowed to stand for 12 hours at 5°. A constant amount of sensitized sheep erythrocytes (1×10^9/ml) was added to each antigen–antibody complex and incubated for 30 minutes at 37°. The effects of peptide fragments, which were mixed with the antibody solution in advance, were also measured by the change of degree of hemolysis at the constant dilutions of *anti*-M_2-cyt. c G-Fract.

Inhibition of cytochrome c-cytochrome oxidase reaction by the antibody was measured as follows. Cytochrome oxidase from *Saccharomyces oviformis* M_2 was isolated and purified following Sekuzu's method.[15] Normal and *anti*-M_2-cyt. c IgG fractions at the same concentration were added to the ferro-cytochrome c solution. After 30 minutes, cytochrome oxidase was added and the decrease in absorbancy at 550 mμ was recorded at 20° in 0.15M phosphate buffer pH 7.0. Effects of the peptide fragments against the inhibitory

7) Y. Baba, H. Mizushima, A. Ito, and H. Watanabe, *Biochem. Biophys. Res. Commun.*, **26**, 505 (1967).
8) R.A. Kekwick, *Biochem. J.*, **34**, 1248 (1940).
9) H.B. Levy and H.A. Sober, *Proc. Soc. Exptl. Biol. Med.*, **103**, 250 (1960).
10) O. Ouchterrolony, *Acta Pathol. Microbiol. Scand.*, **26**, 507 (1949).
11) S.V. Boyden, *J. Exp. Med.*, **93**, 107 (1951).
12) M.M. Mayer, A.G. Osler, O.G. Bier, and M. Heidelberger, *J. Immunol.*, **59**, 195 (1948).
13) Z. Ovary, *Intern. Arch. Alergy Appl. Immunol.*, **3**, 293 (1952).
14) J.A. Kolmer and F. Boerner, Approved Laboratory Technic, New York, D. Appleton-Century. 3rd ed. 1941, p. 618.
15) I. Sekuzu, H. Mizushima, and K. Okunuki, *Biochem. Biophys. Acta*, **85**, 516 (1964).

effect of the antibody were tested as follows. Each peptide fragment was preincubated with the antibody solution at room temperature for 12 hours. The resulting mixture was added to a ferro-cytochrome c solution, and after 30 minutes cytochrome oxidase was added.

Result

Of rabbits immunized with monomeric M_2-cyt. c and with CA-cyt. c, circulating antibodies were appeared after three weeks without exception. By the double diffusion test, a broad indistinct band appeared between M_2-cyt. c and the *anti*-M_2-cyt. c G-Fract. in the agar gel bufferized with veronal buffer pH 8.6 at the range of cytochrome c concentrations of 125 μg/ml—2.0 mg/ml, whereas no precipitin band was observed when a cytochrome c other than M_2-cyt. c was employed as the antigen. Toward the *anti*-CA-cyt. c, CA-cyt. c formed a distinct precipitin line at the range of concentrations of 64—250 μg/ml. M_2-cyt. c also formed a precipitin line toward some of antisera elicited from CA-cyt. c injected rabbit at the range of concentrations of higher than 500 μg/ml. H.H.-cyt. c did not react with either antibody G-Fract.

Cross-reactivities between the two yeast cytochromes c were found by the various methods *in vitro*, that is, in the gel-filtration patterns of soluble antigen-antibody complex at the range of excess antigen (Fig. 1), in the passive hemmaglutination test and in the C.F. test. These results were summarized in Table II.

Furthermore, the PCA reaction, in which most of the non-immunochemical interactions between antigen and antisera is excluded, was also carried out. As shown in Table III, the two yeast cytochromes c cross-reacted each other with their antibodies, but H.H.-cyt. c did not react with these antibodies. Moreover, particularly interesting results were obtained in the PCA inhibition test. An intradermal injection of antigen-antibody complex produced a positive reaction at the sensitized site. This reaction was clearly observed by an intravenous injection of Evans blue dye solution as an indicator. The intensity of the response gradually decreased according to the elapse of the time after the intradermal injection and after 5 hours no response was observed. After this period further intravenous challenge with excess antigen could not induce a positive reaction at the sensitized site where an interaction between antigen and antibody had already existed. When *anti*-M_2-cyt. c antibodies were contacted with CA-cyt. c before the intradermal injection, the PCA reaction against intravenous challenge with M_2-cyt. c was inhibited only slightly as shown in Table IV, while, a challenge with CA-cyt. c was completely inhibited. M_2-cyt. c as a contact antigen for the *anti*-M_2-cyt. c antibodies inhibited completely the PCA

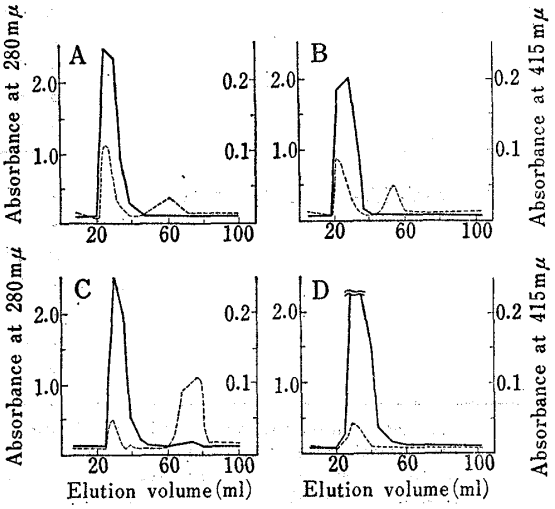

Fig. 1. Gel-filtration of the Soluble Antigen-antibody Complex through Sephadex G-100 (0.9×140 mm). In each case, 2.5 ml of antibody G-Fract. (15—20 mg protein/ml) was mixed with 2.5 ml of antigen (200 μg/ml) or saline. After standing for 24 hours at room temperature, a portion of the mixture was applied on the column equilibrated with saline. Three milliliters fraction were collected at a flow rate of 40 ml/hr. The protein concentration was measured at 280 mμ (solid line), and the concentration of ferro-cytochrome c (antigen) was determined by the absorbance at 415 mμ (broken line) after the addition of 0.5 ml of 0.5% ascorbic acid solution.

A. *anti*-M_2 G.—M_2-cyt. c
B. *anti*-M_2 G.—CA-cyt. c
C. *anti*-M_2 G.—H.H.-cyt. c
D. *anti*-M_2 G.—saline (control)

Table II. Cross-reactivities of *anti*-M_2-cyt. c Antibody against Various Types of Cytochrome c

Antigen	Methods for detection					
	Gel-filt.	C.F.	PCA	Passive hemagglutination	PCA inhibition challanged by	
					M_2-cyt. c	CA-cyt. c
M_2-cyt. c	+	+	+	+	+	+
CA-cyt. c	+	+	+	+	−	+
H.H.-cyt. c	−	−	−	−	−	−

Table III. Cross-reactivities of *anti*-Yeast Cytochromes c Antibodies against Yeast and Horse Cytochrome c in the PCA Reaction

Antibody dilution		Antigen		
		M_2-cyt. c	CA-cyt. c	H.H.-cyt. c
anti-M_2-cyt. c (G Fract.)	× 50	+	+	−
	× 100	+	+	−
	× 200	+	+	−
	× 400	+	+	−
	× 800	+	±	−
	×1600	±	−	−
	×3200	±	−	−
anti-CA-cyt. c (G Fract.)	× 50	+	+	−
	× 100	+	+	−
	× 200	+	+	−
	× 400	+	+	−
	× 800	+	+	−
	×1600	−	±	−
	×3200	−	+	−

The sensitization was carried out by intradermal injection with 0.1 ml of the antibody solution in guinea pigs. Five hours after the sensitization, 1.0 ml of 5% Evans blue solution containing 2.0 mg of antigen was given intravenously. The response was estimated by measuring the length and breadth of the coloured region exactly 60 minutes after the intravenous injection.

(+: more than 10×10, ±: 9×9—5×5, −: less than 4×4)

Five animals were used for each antibody solution.

Table IV. Effects of Yeast and Horse Cytochromes c against *anti*-Yeast Cytochromes c Antibodies in PCA Inhibition Test

1) With *anti*-M_2 G. Fract.

Antigen		Concentrations of contact antigen (μg/ml)											
Contact	Challenge	1000	500	250	125	64	32	16	8	4	2	1	0.5
M_2	M_2	##	##	##	##	##	##	##	##	##	+	+	−
CA	M_2	±	±	±	±	−	−	+	±	−	−	−	−
H.H.	M_2					±	−	−	−	−	−		
M_2	CA	##	##	##	##	##	##	##	##	##	##	##	±
CA	CA		##	##	##	##	##	##	##	##	##	##	−

2) With *anti*-CA G. Fract.

Antigen		Concentrations of contact antigen (μg/ml)											
Contact	Challenge	1000	500	250	125	64	32	16	8	4	2	1	0.5
M_2	CA					±	±	++	++	−	−		
CA	CA		+++	+++	+++	+++	+++	+++	++	++	±	±	
H.H.	CA	±	±	−	−	−	−	−	−				
M_2	M_2		+++		+++		+++		+++		±		
CA	M_2		+++		+++		+++		+++		+++		

The inhibitions were expressed by the percentage decrease in the sum of the length and breadth of blue region caused by the intradermal injection of antibody previously contacted with various concentrations of antigens.
(+++: 100—90%, ++: 89——50%, +: 49—20%, ± 19—10%, and —: 9—0%)
Five test animals were taken in each experiment.

TABLE V. Complement Fixation Levels of M_2-cyt. c and the Peptide Fragments against *anti*-M_2-cyt. c Antibody

Antigen	Minimum concentration for complement fixation Antisera dilution	
	1:10	1:40
M_2-cyt. c	2.0×10^{-2} μmole/ml	1.2×10^{-1} μmole/ml
Peptides mixture of trypsin-treated M_2-cyt. c	5.0×10^{-3}	2.0×10^{-2}
Peptides mixture of chymotrypsin-treated M_2-cyt. c	1.0×10^{-1}	$>4\times10^{-2}$
P-1	nothing	nothing
P-2	nothing[a)]	nothing
P-3	nothing	nothing
P-4	5.0×10^{-3}	2.0×10^{-2} [b)]
P-5	nothing	nothing
P-6	nothing	nothing
P-7	1.0×10^{-2}	nothing

The values were determined by the dose which gave higher than 75% inhibition of hemolysis by a two-dimensional titration method described in the text with the exception in the foot note b).
a) (Nothing) means no inhibitory capacity at the concentration of 2×10^{-2} μmole/ml.
b) Slightly, this value for about 50% inhibition.

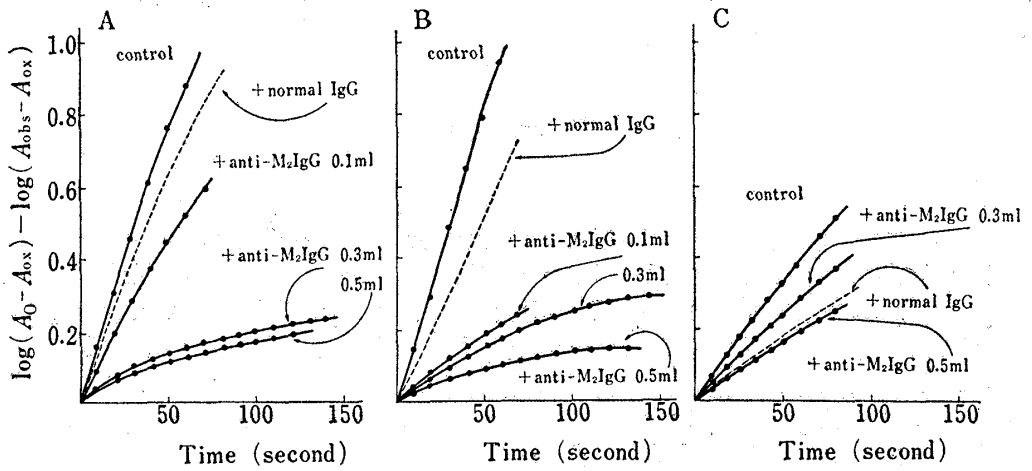

Fig. 2. The Effect of *anti*-M_2 IgG against the Cytochrome c-Cytochrome Oxidase binding. Ferro-cytochrome c (2×10^{-5}M) was added to Cytochrome Oxidase (10^{-8}M)

Previously, ferro-cytochrome c was incubated with *anti*-M_2, or normal IgG solutions (O.D. 280 mμ: 12.27) for 1.5 hours. All experiments were carried out at 20° in 3.0 ml of phosphate-saline buffer pH 7.0. The progress of the enzymatic reactions was estimated by determining the difference of absorbance at 550 mμ between at the initial time (A_o—A_{ox}) and at the scheduled time (A_{obs}—A_{ox}). "Standard" was determined by the reaction between each ferrocytochrome c and cytochrome oxidase without any globulin solution.
A. M_2-cyt. c-cytochrome oxidase B. CA-cyt. c-cytochrome oxidase C. H.H.-cyt. c-cytochrome oxidase

reaction by intravenous challenges of both M_2-cyt. c and CA-cyt. c. A similar response was observed in the tests using *anti*-CA-cyt. c antibodies.

On the other hand, relatively long peptide fragments prepared by enzymatic digestion and by cyanogen bromide cleavage were tested for antigenic activity. In Table V, the results obtained by the C.F. test show that the enzymatic hydrolysate of cytochrome c, especially tryptic hydrolysate were capable of fixing complements with *anti*-M_2-cyt. c antibody in the same level of concentration as is the case of the native cytochrome c. Of isolated peptide fragments which were separated and purified from these hydrolysate, P-4 and P-7 were found to fix complements, although large amounts were necessary, whereas other fragments and shorter peptides failed to bind them. All these fragments did not appear to be an effective inhibitor against the complement fixation by the antigen-antibody complex, but they rather showed additive effects to approximately the same extent.

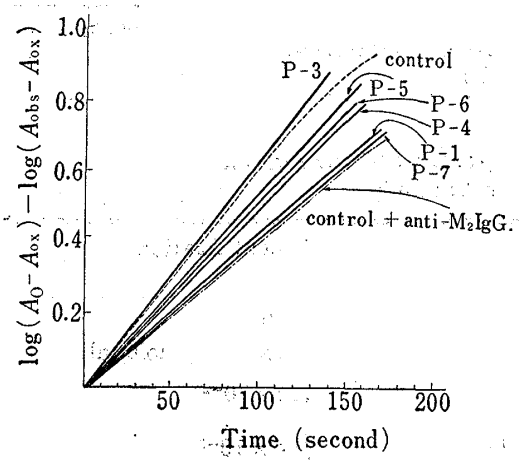

Fig. 3. The elimination of the effect of antibody by the peptide fragments. Enzymatic assays were carried out according to the procedure in Fig. 2, except additional preincubation of anti-M_2 IgG (O.D. 280 mμ: 1.227) with peptide fragments (2×10^{-5}M) for 12 hours at room temperature.

The oxidation of ferro-cytochrome c by cytochrome oxidase was estimated spectrophotometrically by the decrease in absorbance at 550 mμ. *anti*-M_2-cyt. c antibody preincubated with ferrocytochrome c was found to inhibit the oxidation. The extent of inhibition was linear with the increase of antibody amounts (Fig. 2). A similar result was also obtained on the CA-cyt. c-cytochrome oxidase reaction. In contrast, H.H.-cyt. c-cytochrome oxidase interaction was not inhibited by *anti*-M_2-cyt. c antibody. Normal IgG in the same protein concentration showed little inhibitory effect in cytochrome c-cytochrome oxidase interaction. Then, the effects of the peptide of cytochrome c against this inhibitory effect of *anti*-M_2-cyt. c antibody was examined. As shown in Fig. 3, P-3, decapeptide from trypsin digest of M_2-cyt. c, completely, and P-4, P-5 and P-6, partly, eliminated the inhibitory effect of the anti body against the enzymatic reactior

Discussion

The amino acid sequences of more than 30 different species of cytochrome c were already determined, and about 30 amino acid residues on the primary structure are different each other in the two yeast cytochromes c employed in the present study. These variances are markedly localized in three clusters, and the greater part of the peptide chains are covered with the common amino acid sequences. In contrast, the difference in more than 40 amino acid residues between vertebrate and yeast cytochromes c, for example, between H.H.-cyt. c and the two yeast cytochromes c, are scattered throughout the molecules.

The extent of the immunochemical cross-reactivities among these cytochromes c obtained by the various *in vitro* methods appeared to be in parallel with the degrees of sequence differences. However, the possibility was not neglected that there are some non-specific affinities to antibodies due to the basic property of cytochrome c in these conventional methods. The PCA inhibition test, as well as the PCA test provided more reliable results for the detection of small immunological differences between closely related proteins. It has been shown by

Janoff, *et al.*[16] that the antibody neutralized prior to contact with antigen fails to give a positive PCA reaction. When antigen is contacted with antibody, if there is any interaction between them, the permeability of the capillary vessels increases immediately after the injection of this antigen-antibody mixture. This increase is transient, however, and the response is gradually vague according to the elapse of the time after the intradermal injection. After more than 4 hours of further challenge by an intravenous injection of excess antigen cannot induce any response in the skin of the guinea pig which was already given an intradermal injection of the complex. Therefore, it seems to be useful to apply this reaction for the detection of minor cross-reactive antibodies against the various antigens which resemble to the antigen used for immunization.

The results suggest that several antigenic determinants exist in both types of yeast cytochrome c, which are shared with M_2-cyt. c and CA-cyt. c, but not with H.H.-cyt. c.

Immunochemical reactivities of the peptide fragments derived from M_2-cyt. c were examined first by the C.F. inhibition test. It was shown that none of them were effective inhibitors, but rather play additively in fixing complements with the M_2-cyt. c-*anti*-M_2-cyt. c antibody complex. P-4 and P-7 appeared to be more potent immunochemically to fix complement considering the result in the C.F. test without the native antigen.

On the other hand, considering from the fact that the specific antibody inhibited the cytochrome c-cytochrome oxidase interaction, it seemed to be likely that some of antigenic determinants of cytochrome c were located near its binding site with cytochrome oxidase. H.H.-cyt. c, which could not bind the *anti*-yeast cyt. c antibody, was still active as substrate for cytochrome oxidase after treatment with *anti*-M_2-cyt. c antibody. This inhibitory effect of the antibody was eliminated by the addition of peptide fragments P-3, P-4, P-5 and P-6. The difference between P-3, which showed complete elimination, and other partly effective fragments may be explained by the difference in their affinity to the oxidase molecule.[7] As P-3 showed little effect against cytochrome c-cytochrome oxidase interaction, oxidase molecule may contact with cytochrome c without any affection by the P-3-antibody complex. In contrast, in the case of P-4, results cannot simply be explained. Because it may be that P-4 has an affinity toward both antibody and cytochrome oxidase molecules.[7]

According to the three-dimensional structure of cytochrome c by X-ray crystallography the sequences including P-3 extend over the surface of the molecule aside from the "crevice".[17,18] The region including P-3 may be playing a role in binding with antibody.

From the recent studies on the immunochemical cross-reactivities among vertebrate cytochromes c, a single amino acid substitution at the position 58 was proposed to be important for antigenic determinant.[2] Of three clusters where amino acid variances are localized, differences between the two yeast cytochromes c are involved in the middle part, particularly the position from 53 to 63. Considering from the results in cross-reactivities, other different residues at the position 20, 22, 37, 66, 88, 98 and 100 which are common between M_2-cyt. c and H.H.-cyt. c do not appear to be concerned with antigenic determinants.

Of many reports on the antigenicity of various proteins, a single peptide with a full antigenicity of the whole protein was not successfully obtained except for tobacco mosaic virus protein.[19] In the present study, within the limits of cleavage characterized by the specificity of proteolytic enzymes and cyanogen bromide, any single peptide fragment could not fully cover the antigenic determinant of the M_2-cyt. c.

16) A. Janoff, W. Benjamin, B. Zweifach, A.L. Nagler, and Z. Ovary, *Circulat. Res.*, **9**, 407 (1961).
17) T. Takano, R. Swanson, O.B. Kallai, and R.E. Dickerson, Cold Spring Harbor Symposia on Quantitative Biology," Vol. 36, 1972, p. 397.
18) R.E. Dickerson, T. Takano, D. Eisenberg, O.B. Kallai, L. Swanson, A. Cooper, and E. Margoliash, *J. Biol. Chem.*, **246**, 1511 (1971).
19) J.M. Stewart, J.D. Young, E. Benjamini, M. Shimizu, and C.Y. Leung, *Biochemistry*, **5**, 3396 (1966).

From the results presented here and by the comparison of sequencial composition among various cytochromes c, it may be suggested that the polypeptide chain from 51 to 60, which stretches over P-4 and P-5 seems to be a significant region of the antigenic determinant of cytochrome c, whereas a direct evidence was not obtained yet.

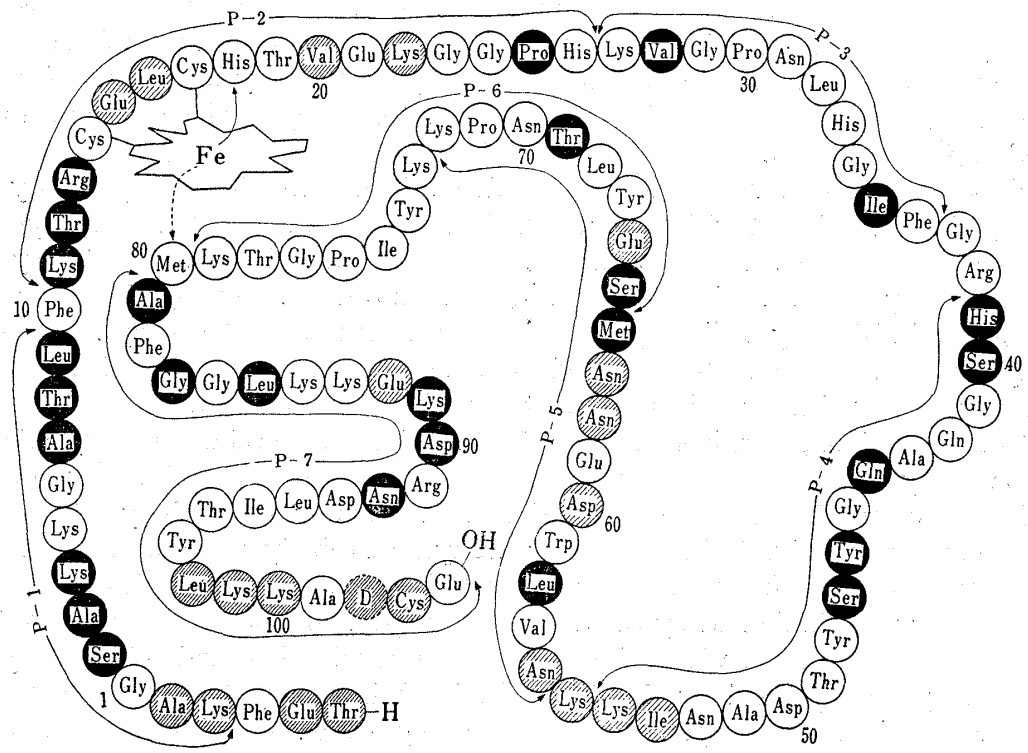

Fig. 4. The Amino Acid Sequence of M_2-cytochrome c Polypeptide Chain. White circles represent common amino acid loci among the three, *i.e.*, M_2, CA and H.H. cytochromes c. Black circles represent common amino acid loci between M_2 and CA cytochromes c, but different between yeast and H.H. cytochromes c. Shaded circles represent different loci among the three cytochromes c. These data were obtained from the results of Narita[20,21] and Margoliash.[22]

Acknowledgement The authors would like to thank Dr. Hiroshi Nakamura for stimulating discussions. We are also grateful for the excellent technical assistance for Miss Tokuko Satoh and Miss Itsuyo Sekine.

20) Y. Yaoi, K. Titani, and K. Narita, *J. Biochem.* (Tokyo), **59**, 247 (1966).
21) K. Narita and K. Titani, *Proc. Japan Acad.*, **41**, 831 (1965).
22) E. Margoliash, E.L. Smith, G. Kreil, and H. Tuppy, *Nature*, **192**, 1125 (1961).