Fraction 16–20 was eventually purified from the accompanying disulphide by preparative TLC on cellulose with n-propanol-1 N HCl (3:2) to give a 2:1 mixture of **3b** and **3c** as determined by PMR spectroscopy (in 2 N DCl).

B) *Tyrosinase oxidation of secoadenochromines and 5-thiolhistidine.* A mixture of secoadenochromine A (**3a**, 6.63 mg; 0.0125 mmoles) and 5-thiol-L-histidine dihydrochloride (7 mg; 0.025 mmoles) in phosphate buffer, pH 6.8, was oxidized in the presence of mushroom tyrosinase (2.2 mg) at room temperature. After 2.5 h the oxidation was stopped by acidification. Fractionation of the reaction mixture on Dowex 50 WX2 (200–400 mesh, H^+ form) gave: the disulphide of 5-thiolhistidine (~ 2 mg); adenochromine A (**1a**, 1.1 mg, 12%) and **1b** (0.2 mg, 2.16%) which were separated by preparative TLC on cellulose with n-propanol-1 N HCl (3:2). Similar results were obtained in the enzymic oxidation of a mixture of secoadenochromines B (**3b**) and C (**3c**), in a molar ratio 4:1, leading to: adenochromine B (**1b**, 1.7%); adenochromines A and C (**1a** and **1c**, 7%) along with 82% of unreacted material.

Results and discussion. Under biomimetic conditions, the reaction of 5-thiolhistidine with dopaquinone, generated by tyrosinase oxidation of dopa, proceeds smoothly and clearly to give, along with a small amount (3%) of the 3 adenochromines, the parent monoadducts secoadenochromines A, B and C in 55, 20, 10% yields, respectively.

Attempts to increase the yields in adenochromines under different conditions, or with a crude preparation of *Octopus* tyrosinase[8], were unsuccessful because of the inability of the enzyme to oxidize efficiently the intermediary secoadenochromines into the corresponding o-quinones. It is relevant, however, that oxidation of secoadenochromines, in the presence of excess of 5-thiolhistidine, gave a 10% yield of adenochromines. In spite of the fact that under in vitro conditions the formation of monoadducts **3** prevails, these experiments are consistent with the view that the formation of adenochromines in *Octopus* may be regarded as a result of deviation of the normal eumelanin pathway[6], involving a nonenzymic reaction between dopaquinone (enzymically produced) and 5-thiolhistidine. The analogy of such a process with phaeomelanin biosynthesis is remarkable, especially when compared with the early stages leading to the formation of 5-S-cysteinyldopa (**4a**) and 2-S-cysteinyldopa (**4c**), as well as 2,5-dicysteinyldopa (**2**). A major difference between the addition of 5-thiolhistidine and cysteine to dopaquinone is that in the former case the addition takes place to a significant extent (about ¼) at C-6 position (by 1,4-addition), which accounts for the in vivo formation of all the 3 possible isomers of **1**.

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8 An active preparation of *Octopus* tyrosinase could be easily obtained after finding that the enzyme is present in the supernatant liquid of the ink sac after spinning off the melanin. Details of the preparation will be described elsewhere.

On the relationship of hemoglobin oxidation with the conformation of hemoglobin

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Summary. The rate of hemoglobin oxidation by various oxidants was studied under aerobic and anaerobic conditions, and the mechanism of hemoglobin oxidation was discussed in relation to the conformation of hemoglobin.

Recent advances on stereochemistry of hemoglobin revealed the allosteric behavior of this protein, which is characterized by the 2 conformational models including the oxy or R state and the deoxy or T state[1-3]. The shift of the R to the T state has been shown to be induced by the binding of organic phosphates such as 2,3-diphosphoglycerate (2,3-DPG) and inositol hexaphosphate (IHP)[4-7].

On the other hand, although the oxidation of hemoglobin by various oxidants has been studied under various conditions, few studies have so far been carried out from the standpoint of the conformation of hemoglobin. With regard to oxidation of hemoglobin by ferricyanide and nitrite and autoxidation of this protein, the effect of 2,3-DPG is mentioned in relation to the oxygen affinity of hemoglobin[8-10]. Recently we investigated the effect of IHP, as a stronger effector, on the redox reaction of this hemoprotein by some oxidoreductants and showed that the redox reaction of this protein may be affected by the conformational changes due to the binding of IHP[11,12]. In this paper, reaction mechanism of hemoglobin oxidation by several oxidants is surveyed in relation to hemoglobin conformation including the R and T state.

Methods. Hemoglobin A, obtained from fresh human red cells by hemolysis was passed through Sephadex G 25 (fine) which was previously equilibrated with 0.05 M bis-tris buffer (pH 7.0) with 0.1 M NaCl. By this procedure, hemoglobin free from organic phosphates was obtained. Catalase-free hemoglobin was prepared according to the method of Huisman and Dozy[13]. Experiments were performed as follows. A 0.2-ml sample of hemoglobin solution (25 μM as hemoglobin tetramer) and 2 ml of 0.05 M bis-tris buffer (pH 7.0) containing 0.1 M NaCl were mixed with or without IHP (90 μM). After mixing, and 5 min standing, the reaction was started by the addition of 20 μl solution of oxidants such as ferricyanide, hydroxylamine, chlorate, H_2O_2, β-naphthoquinone-4-sulfonate or nitrite and the rate of hemoglobin oxidation was spectrophotometrically measured at 25 °C by following the increase in absorbance at 630 nm. The catalase-free hemoglobin was used for the experiment with H_2O_2. The measurement of the rate of

Rate of hemoglobin oxidation by various oxidants under aerobic and anaerobic conditions. The rate of hemoglobin oxidation by ferricyanide, hydroxylamine, H_2O_2, β-naphthoquinone-4-sulfonate, and autoxidation was expressed as μM heme/min. The rate of hemoglobin oxidation by chlorate and nitrite was expressed as $t_{1/2}$ (half time), since the reaction proceeded sigmoidally

	Final concentrations of oxidants	Aerobic IHP (−)	IHP (+)	Anaerobic IHP (−)	Temperature (°C)
Ferricyanide	45 μM	20.0 μM/min	195.0 μM/min	very fast	25
		1.8 μM/min	22.0 μM/min	very fast	4
Hydroxylamine	900 μM	13.5 μM/min	22.0 μM/min	52 μM/min	25
Chlorate	110 mM	52 min	27 min	13 min	25
H_2O_2	140 μM	12.2 μM/min	43.8 μM/min	very fast	25
β-Naphthoquinone-4-sulfonate	900 μM	16.9 μM/min	146.0 μM/min	very fast	25
Autoxidation		1.8 μM/15 min	9.6 μM/15 min		38
Nitrite	900 μM	5.2 min	35 min	very slow	25

hemoglobin oxidation under anaerobic conditions was performed in a Thunberg-type quartz cell after replacement of air with Q gas (helium/isobutane, 99.05:0.95). The experiment of autoxidation was performed at 38 °C and the changes in absorbance were pursued at 578 nm.

Results and discussion. The mechanism of hemoglobin oxidation by various oxidants was studied under aerobic conditions with or without IHP, and under anaerobic conditions without IHP. The oxidation rate by ferricyanide, hydroxylamine, chlorate, H_2O_2, β-naphthoquinone-4-sulfonate and nitrite under these conditions is summarized in the table. The oxidation of hemoglobin by these oxidants was classified into 2 groups with regard to the reaction mechanism. Ferricyanide, hydroxylamine, chlorate, H_2O_2 and β-naphthoquinone-4-sulfonate belonged to the 1st group. In this group, the acceleration of the hemoglobin oxidation by these reagents was observed as hemoglobin was bound with IHP and deoxygenated. Since the structural change in oxyhemoglobin from the R to the T state occurs by deoxygenation[14] and is probably induced by the binding of IHP to oxyhemoglobin, it may be possible to say that the oxidation rate by these oxidants was accelerated as the fractions of the T state hemoglobin were increased. The oxidation of hemoglobin by ferricyanide was also accelerated as much as 12 times in the presence of IHP at 4 °C. Autoxidation of hemoglobin might also be involved in the 1st group as far as the reaction mechanism is concerned, since it is well known that autoxidation is considerably accelerated in accordance with the decrease in oxygen concentration[15].

The course of oxidation of hemoglobin by nitrite seems to be different from that by the oxidants stated above and belongs to another category. The rate of oxidation by this reagent was accordingly decreased as hemoglobin was liganded with IHP and when deoxygenated. These results suggest that the R state of hemoglobin is favored for the oxidation of this protein by nitrite in preference to the T state.

Although the differences in the reaction mechanism of hemoglobin oxidation by various species of oxidants under aerobic and anaerobic conditions have so far been considered due to the properties of oxidants, our results suggest that the differences in the reaction mechanism may be essentially due to hemoglobin itself, which is equilibrated between the R and the T state with its quaternary structure.

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Determinant of efficiency of a monomeric enzyme: Acceleration by site-specific molecules for trypsin

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Summary. The interaction of a specific ligand at substrate binding site was shown to be responsible for the catalytic efficiency of trypsin. The reasoning of 'induced fit' theory was refined by kinetic analysis of characteristic properties of 'inverse' substrates.

It is generally believed that the endopeptidase, trypsin, and chymotrypsin[2] hydrolyze only those substrates which contain specificity-determining functional groups in their carbonyl side of the esteric or amide structure. However, in the preceding paper[3], we have reported that a certain class of compounds **1**, in which the arrangement of the site-specific group is of the 'inverse' type of the normal substrates of trypsin **2**[4] (fig. 1), e.g., a cationic center is included in the leaving group instead in the acyl moiety, are susceptible to the enzymatic hydrolysis as well. In this report we demonstrate that the site-specific cationic molecules enhance the efficiency of the deacylation process as a characteristics of 'neutral' acyl trypsin derived from the 'inverse substrates' providing possible novel examples of 'induced fit' concept.

During the course of the tryptic hydrolysis of these substrates, ES complex formation and acylation proceed rapid-