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A REACTION CELL FOR SIMULTANEOUS MEASUREMENTS OF FLUORESCENCE AND ABSORPTION IN A HEMOGLOBIN SOLUTION AT VARIOUS OXYGEN PRESSURES

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SUMMARY: An apparatus was constructed to carry out measurements of fluorescence, optical absorption and oxygen partial pressure in a hemoglobin or other solution simultaneously, and its performance was examined. This apparatus has a rhombiform optical cell in place of the usual square optical cell used in commercially available spectrofluorometers. Fluorescence emitted at the region near the cell surface in the solution could be detected satisfactorily and easily even if the solution had strong light absorption bands at both the excitation and the emission wavelengths in the presence of high concentrations of a chromophore. This apparatus was particularly effective for studies on the interactions of a fluorescent allosteric effector with hemoglobin at various degrees of deoxygenation. Consequently, it was proved experimentally that the fluorescence of β-naphthyl triphosphate bound to hemoglobin is completely quenched. Moreover, simultaneous and continuous measurements of the oxygen-binding equilibrium of hemoglobin and the allosteric effector-binding to hemoglobin as a function of oxygen partial pressure could be satisfactorily carried out, and it is confirmed that β-naphthyl triphosphate binds not only to deoxyhemoglobin but also to fully oxygenated hemoglobin and lowers strongly the oxygen affinity of hemoglobin as an allosteric effector.

Simultaneous measurements of both fluorescence and optical absorption are sometimes very important in studies on the interaction of two kinds of proteins or the interaction of a protein with its ligand. Analysis of the correlation of the changes in the fluorescence and the optical absorption might also frequently be valuable for elucidating the molecular mechanism of the interaction. However, if the protein or its ligand has strong optical absorption bands at the excitation and the emission wavelengths, the square optical cell used in commercially available spectrophotometer is inappropriate, since fluorescence emitted in the cell will be severely attenuated due to the absorption during its propagation. On the other hand, by employing a method to detect fluorescence at the front of the usual square optical cell, a sufficient fluorescence intensity can be obtained, since no or only a very slight amount of fluorescence emitted at the region near the cell surface is absorbed by the chromophore in the cell. However, the technical difficulty of obtaining a suitable geometrical arrangement of excitation light source and fluorescence detector remains.

We devised a reaction cell having a rhombiform optical cell compartment with

*Abbreviations used: HbA, human hemoglobin; DPG, 2,3-diphosphoglycerate; IHP, inositol hexaphosphate; β-napP_3, β-Naphthyl triphosphate; P_{50}, half saturated oxygen partial pressure.

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which a sufficient fluorescence intensity is obtainable even in the presence of a high concentration of a chromophore and with which optical absorption can also be simultaneously determined. The rhombiform optical cell compartment can be mounted in place of the usual square optical cell without any modification of commercially available spectrofluorometers. Moreover, the light path length is variable within 15 mm so that both the fluorescence and optical absorption of a highly concentrated solution can be measured.

This apparatus was applied to investigate the interactions between a fluorescent organic phosphate, β-naphthyl triphosphate (β-$NapP_3$) , and human hemoglobin (HbA) at various degrees of oxygen partial pressure. Previously, Tyuma et al. reported that the binding of allosteric effectors such as 2,3-diphosphoglycerate (DPG) and inositol hexaphosphate (IHP) to hemoglobin during its deoxygenation could be indirectly estimated[1,2] by the combined use of oxygen equilibrium curves and Adair's successive oxygenation theory[3]. This paper describes the first direct and continuous measurements of the binding of an allosteric effector to HbA during its deoxygenation.

MATERIALS AND METHODS

1. Optical cell compartment of the reaction cell: The optical cell compartment of our reaction cell is equipped with two quartz blocks, which are essential for simultaneous measurements of fluorescence and optical absorption. The solid line shows the incident excitation light beam, and the broken and dotted lines show the transmitted and emitted light, respectively. Fluorescence intensity is measured at right angles to the excitation light beam. In this method, sufficient fluorescence can be emitted from the region near the cell surface in the direction of the fluorescence detector even if the solution has strong absorption bands at the excitation and emission wavelengths. In order to avoid the detection of directly reflected excitation light, the angle, shown in Fig.1A, is not 45 degrees but 52.4 degrees. Since the shape of the solution enclosed by the two quartz blocks is rhombic, we named this cell a "rhombiform optical cell".

2. Apparatus: A diagram of our apparatus is shown in Fig.1B, and a sketch of the reaction cell with optical cell compartment is shown in Fig.1C.

Fluorescence intensity measurements were performed at the excitation wavelength of 285 nm and the emission wavelength of 340 nm. At the excitation wavelength of 285 nm, the fluorescence intensity of β-$NapP_3$ is nearly maximum. At 340 nm, the emission fluorescence intensity is nearly maximum and the absorption of oxyHbA and deoxyHbA are almost identical. The HbA solution in the absence of β-$NapP_3$ was also examined fluorophotometrically at the same wavelengths.

Absorption measurements were performed at 542 nm, and absorption changes were used for determination of the percent saturation of HbA with oxygen. As we used a commercial spectrofluorometer, we combined a mirror(l) and an optical fiber(m) to guide the transmitted light to the photomultiplier(n), which was located at the outside of the sample room(s).

Oxygen partial pressure was determined by means of a Clark oxygen electrode (model 4004, Yellow Spring Co. Inc.), which was directly immersed in the HbA solution. The solution was gently stirred with a magnetic stirring bar placed under the oxygen electrode during the experiments.

A Shimadzu RF-501 recording spectrofluorometer was used for fluorophotometric measurements, and also for spectrophotometric measurements. Fluorescence and absorbance were recorded against oxygen partial pressure by the use of a 2-pen X-Y recorder (model F-72T, Riken Denshi). A suitable volume of sample solution is 3.5 ml. The temperature of the sample solution was controlled at 25°C by means of

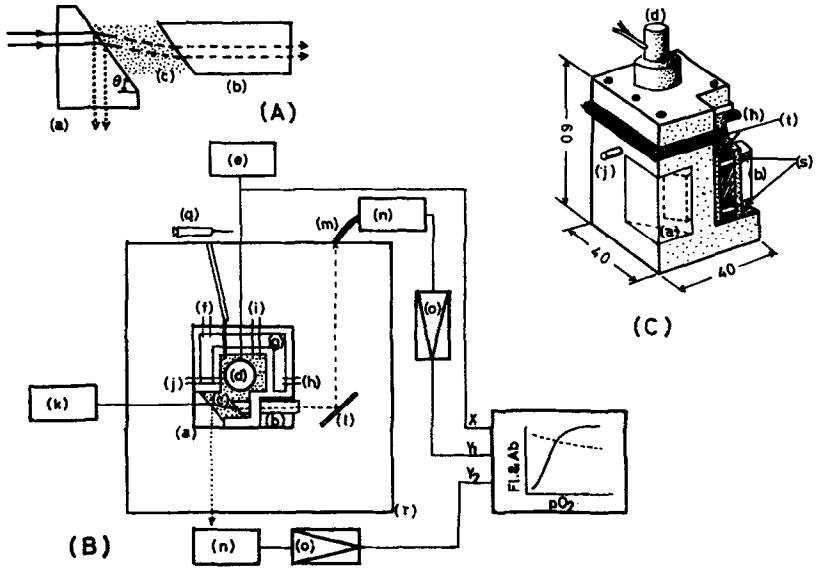

Fig.1. (A) Optical cell compartment of the reaction cell. The solid line shows the incident excitation light beam, and the broken and the dotted lines show the transmitted and the emitted light, respectively. Fluorescence is detected at right angles to the excitation light beam. Since θ is 52.4 degrees, light directly reflected from the excitation light beam is avoided. Two blocks, (a) and (b), are made of quartz. (c); hemoglobin solution. (B) Diagram of our apparatus. (d), oxygen electrode; (e), d.c. power supply; (f), water inlet; (g), water jacket through which temperature-controlled water flows to the bottom part of the reaction cell; (h), water outlet; (i), inlet for nitrogen, oxygen gas or air; (j), outlet for gases; (k), light source and monochromator; (l), mirror; (m), optical fiber; (n), photomultiplier for the transmitted or emitted light; (o), amplifier for the photomultiplier output; (p), 2-pen X-Y recorder; (q), injection syringe; (r), the sample room of spectrofluorometer. (C) Brief sketch of our reaction cell with optical cell compartment. Lengths are shown in millimeters. (s), screws to change light path length; (t), silicon rubber packing to keep the inside of the reaction cell air-tight.

a water jacket(g) with a Taiyo Coolnit CL-15 circulating water system. The syringe(r) was used for the injection of concentrated IHP solution at the completely deoxygenated stage.

3. Materials: Stripped HbA was obtained by the method described previously[4], and the concentration was accurately determined using a molar extinction coefficient E=56,800 at 542 nm for oxyHbA[5]. β-Naphthyl oligophosphates were synthesized by the method of Kuwajima and Asai[4], and β-NapP_3 was used in these experiments. The concentration was estimated on the basis of a molar extinction coefficient E=4,600 at 274 nm and pH 7.0. IHP was obtained from ICN Pharmaceuticals Inc.

RESULTS AND DISCUSSION

1. Fluorescence of oxyHbA: The fluorescence intensity of oxyHbA was measured at the excitation wavelength of 285 nm and at the emission wavelength of 340 nm by the use of the reaction cell with rhombiform optical cell compartment. A comparison of the fluorescence intensity measured from the side of a usual square optical cell with that from the rhombiform optical cell is presented below.

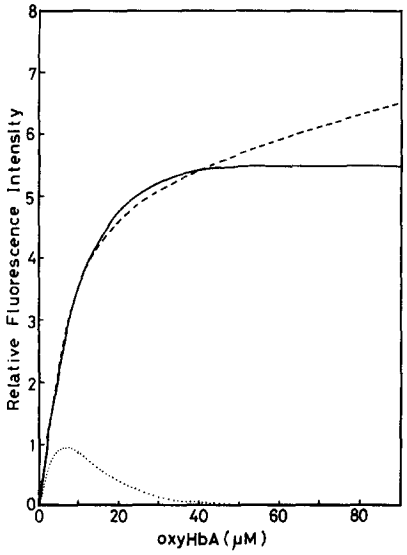

Fig.2. Fluorescence of oxyHbA. Fluorescence of oxyHbA detected from the side of a usual square optical cell (the dotted line), and from the rhombiform optical cell compartment (the broken line). Theoretical fluorescence intensity obtainable from the front of a usual square optical cell (the solid line). Experimental conditions: pH 7.4 (0.05 M bis-tris HCl), 25°C; the total Cl^- concentration was 0.1 M.

The fluorescence intensities obtained from the side of a square optical cell (Fig.2,A) increased at first in proportional to the concentration of oxyHbA, but soon decreased with increasing concentration of oxyHbA because of absorption by oxyHbA at the emission wavelength. The fluorescence was undetectable at concentrations of HbA higher than 50 μM.

In contrast, the fluorescence intensities from the rhombiform optical cell (Fig.2,B) at concentrations higher than 10 μM oxyHbA were much larger than those from the side of a square optical cell. This is, of course, because the fluorescence emitted near the cell surface of the rhombiform cell as well as that emitted near the front cell surface of a usual square cell is satisfactorily detectable without significant absorption even in the case of a highly concentrated oxyHbA solution. The fluorescence intensities from the front of a square optical cell (Fig.2,C) were calculated numerically on the basis of a theoretical equation derived by Forster[6].

2. Fluorescence of β-$NapP_3$ in oxyHbA: In 1975, Kuwajima et al.[4] showed that β-$NapP_3$ binds to bovine oxyhemoglobin in the absence of bis-tris buffer, and that the bound β-$NapP_3$ is released by the addition of sufficient amounts of IHP. The fluorescence intensities in the presence or in the absence of β-$NapP_3$ from the rhombiform optical cell and from the side of a usual square optical cell as a function of oxyHbA concentration are shown in Fig.3.

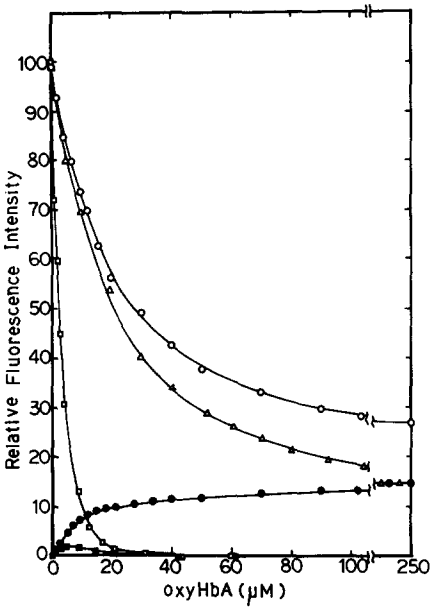

Fig.3. Fluorescence of β-$NapP_3$ in oxyHbA. Concentrated oxyHbA (450 μM tetramer) solution were titrated against buffer solution containing 2 mM IHP(●,■), and against buffer solution containing 2 mM IHP and 57 μM β-$NapP_3$(○,□). ■,□ ; the fluorescence from the side of a usual square optical cell, ●,○ ; from the rhombiform optical cell compartment. △; the fluorescence of the oxyHbA solution containing 57 μM β-$NapP_3$ in the absence of IHP by the use of rhombiform optical cell compartment. All solution conditions were the same as in Fig.2.

A buffer solution containing 57 μM β-$NapP_3$ and 2 mM IHP was first prepared, and titrated with a concentrated oxyHbA solution(450 μM tetramer). Fluorescence was undetectable from the side of a square optical cell at concentrations of oxyHbA higher than 50 μM. Sufficient fluorescence was, however, detected by the use of the rhombiform optical cell even if an oxyHbA concentration higher than 100 μM was used.

The fluorescence intensities of the oxyHbA solution containing 57 μM β-$NapP_3$ in the absence of IHP are smaller than those in the presence of IHP, since β-$NapP_3$ binds to oxyHbA. The fluorescence intensity of β-$NapP_3$ containing an enough amount of oxyHbA agreed well with that of oxyHbA itself in the absence of β-$NapP_3$. This data shows that the fluorescence of the bound β-$NapP_3$ to oxyHbA is completely quenched by the heme group. On the basis of the large quenching radius (42 Å) of the heme group[7], Kuwajima et al.[4] assumed that bound β-naphthyl oligophosphate is completely quenched. By the use of our reaction cell, it was proved that the their assumption of complete quenching of fluorescent organic phosphate bound to Hb is reasonable. This result cannot be obtained by the use of a usual square optical cell, since the fluorescence emitted in the cell is absorbed during its propagation.

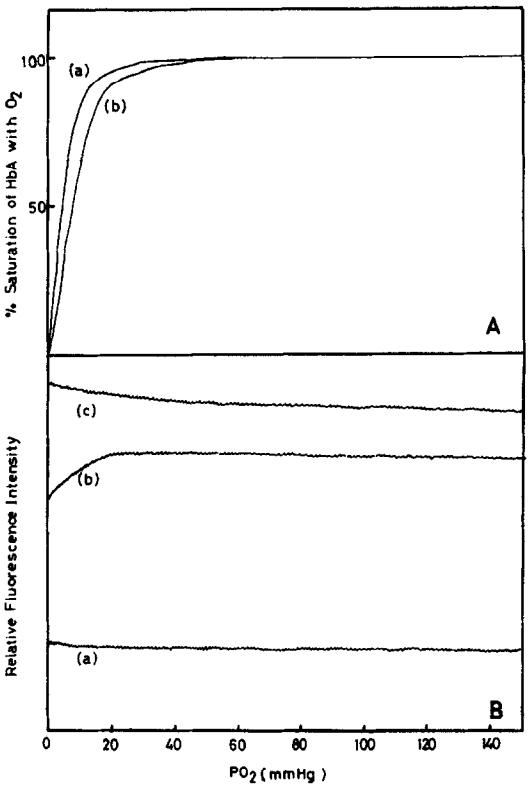

Fig.4. Fluorescence and absorption changes accompanying the deoxygenation of HbA. A, oxygen equilibrium curves; B, fluorescence changes against oxygen partial pressure; (a), 30 μM HbA alone; (b), in the presence of 60 μM β-$NapP_3$; (c), in the presence of 60 μM β-$NapP_3$ and 2 mM IHP.

In preliminary experiments with our reaction cell, it was also found that the fluorescence intensities were proportional to the concentration of free β-$NapP_3$ up to at least 100 μM oxyHbA solution. Thus, the difference of fluorescence intensities in the presence and in the absence of β-$NapP_3$ is a measure of free β-$NapP_3$ concentration at each concentration of oxyHbA. The difference fluorescence intensities obtained from the side of a square optical cell became undetectable at concentrations of oxyHbA higher than 50 μM. In practice, it is difficult to measure the fluorescence changes in hemoglobin solutions at concentrations higher than 20 μM with a commercial spectrofluorometer. In contrast, sufficient fluorescence of free β-$NapP_3$ can be obtained from the rhombiform optical cell so that the fluorescence changes accompanying effector binding to HbA can be easily measured.

3. Fluorescence and absorption changes accompanying deoxygenation: Firstly, the fluorescence and absorption changes of HbA solution that accompany its deoxygenation in the absence of β-$NapP_3$ were simultaneously measured, as shown in

Fig.4,a. The fluorescence was, however, almost the same at various levels of deoxygenation. Analysis of the absorption changes at various oxygen partial pressures provides the oxygen equilibrium curve of HbA, and P_{50} was evaluated as 4.5 mmHg. This value coincides well with that of other investigators[8] under the same experimental conditions.

Subsequently, the fluorescence and absorption of the HbA solution at various oxygen partial pressures in the presence of 60 μM β-NapP_3 were also examined (Fig.4,b). P_{50} was determined to be 7.5 mmHg, which showed that β-NapP_3 lowered the oxygen affinity of HbA. The fluorescence first increased slightly with deoxygenation, but decreased remarkably at oxygen partial pressures lower than about 30 mmHg. In order to release β-NapP_3 from HbA, the injection syringe was used to add sufficient amounts of IHP (50 mM,0.2 ml) to the same HbA solution, which contained 60 μM β-NapP_3 and was already completely deoxygenated. The fluorescence intensity of the free β-NapP_3 gradually decreased with increasing oxygen partial pressure of the solution. This fluorescence decrease is evidently due to quenching of the free β-NapP_3 by oxygen molecules (Fig.4,c). Thus, the difference fluorescence intensity, (c)-(a), is indicative of the total free β-NapP_3 concentration at each oxygen partial pressure, and (c)-(b) is indicative of bound β-NapP_3 concentration. It is, thus, evident that deoxygenation of HbA at oxygen partial pressures lower than 30 mmHg causes significant binding of β-NapP_3 to the deoxyHbA.

Since the discovery of the remarkable effect of organic phosphates, especially DPG, in lowering the oxygen affinity of HbA[9], much attention has been directed to the mechanism and physiological role of the phenomenon[9,10,11]. In 1968, Benesch et al. showed by direct ultrafiltration measurements that one mole of DPG binds to one mole of tetrameric deoxyHbA, but not to oxyHbA under physiological conditions[12]. In this study, it was found that β-NapP_3 as well as IHP binds not only to deoxyHbA but also to fully oxygenated HbA, and lowers the oxygen affinity of hemoglobin as an allosteric effector.

We have devised an apparatus with which the binding of an allosteric effector to Hb can be directly and continuously measured, and have examined its performance by the use of a fluorescennt analog. The binding features that accompany deoxygenation can be clearly observed, as shown in Fig.4. This apparatus should be readily applicable to precise analysis of the effects of DPG or IHP on the oxygen affinity of Hb in the intermediate stages of deoxygenation, on the assumption of competitive binding with the fluorescent analog.

In summary, our newly devised apparatus offers the following advantages: (1) fluorescence, absorbance and oxygen partial pressure can be measured simultaneously; (2) satisfactory fluorescence measurements can be made even if a fluorescent substance and a dye are both present in a solution which has strong absorption bands at both the excitation and the emission wavelengths; (3) absorp-

tion measurements can also be carried out easily over a comparatively wide range of concentrations; (4) commercial spectrofluorometers can be used without any modifications.

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