Biophysical Chemistry, 44 (1992) 209–217
Elsevier Science Publishers B.V., Amsterdam

BIOCHE 01691

Chlorpromazine induced aggregation of normal human hemoglobin. Electron microscopic evidence

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(Received 24 January 1992; accepted in revised form 3 June 1992)

Abstract

Normal human hemoglobin exceeding a certain minimum concentration (called critical aggregation concentration) undergoes aggregation in presence of the psychotherapeutic drug chlorpromazine (CPZ). The critical aggregation concentration decreases with the increase of CPZ concentration. Electron micrographs of CPZ-treated hemoglobin clearly indicate that the aggregates of hemoglobin are in filamentous form of average width 75 ± 8 Å. A possible mechanism for such aggregation has been discussed.

Keywords: Chlorpromazine; Hemoglobin; Induced aggregation

1. Introduction

Chlorpromazine (CPZ), a non-planar tricyclic phenothiazine group of drug is widely used as an antidepressant and tranquiliser in the treatment of psychotherapeutic patients all over the world. Interest in the studies on the interaction of this drug with different functional macromolecules stems from the fact that the drug, although used for therapeutic purpose, has some undesirable side-effects [1] the underlying mechanisms of which are still not clear. The drug binds with DNA [2] and different proteins [3,4]. A substantial fraction of the drug, used at therapeutic dose, binds with red blood cells (RBC) of patients when administered and gets incorporated into the cells [5]. The concentration of CPZ within RBC of patients that were administered 900 mg of CPZ as daily therapeutic dose, was found to be 360 μM [6]. All these reasons have evoked our interest to study more closely the interaction of this drug with hemoglobin (which is the major protein component of RBC and is the carrier of oxygen from lungs to different tissues).

We have already shown in earlier studies [7,8] that CPZ binds with normal human hemoglobin in a positive cooperative mode with overall binding affinity constant equal to $3.8 \times 10^3\ M^{-1}$, and that such binding leads to release of oxygen from hemoglobin with the associated conformational change in the globin part of the hemoglobin molecule. Our observation on the release of oxygen from drug-treated hemoglobin [7] signifies

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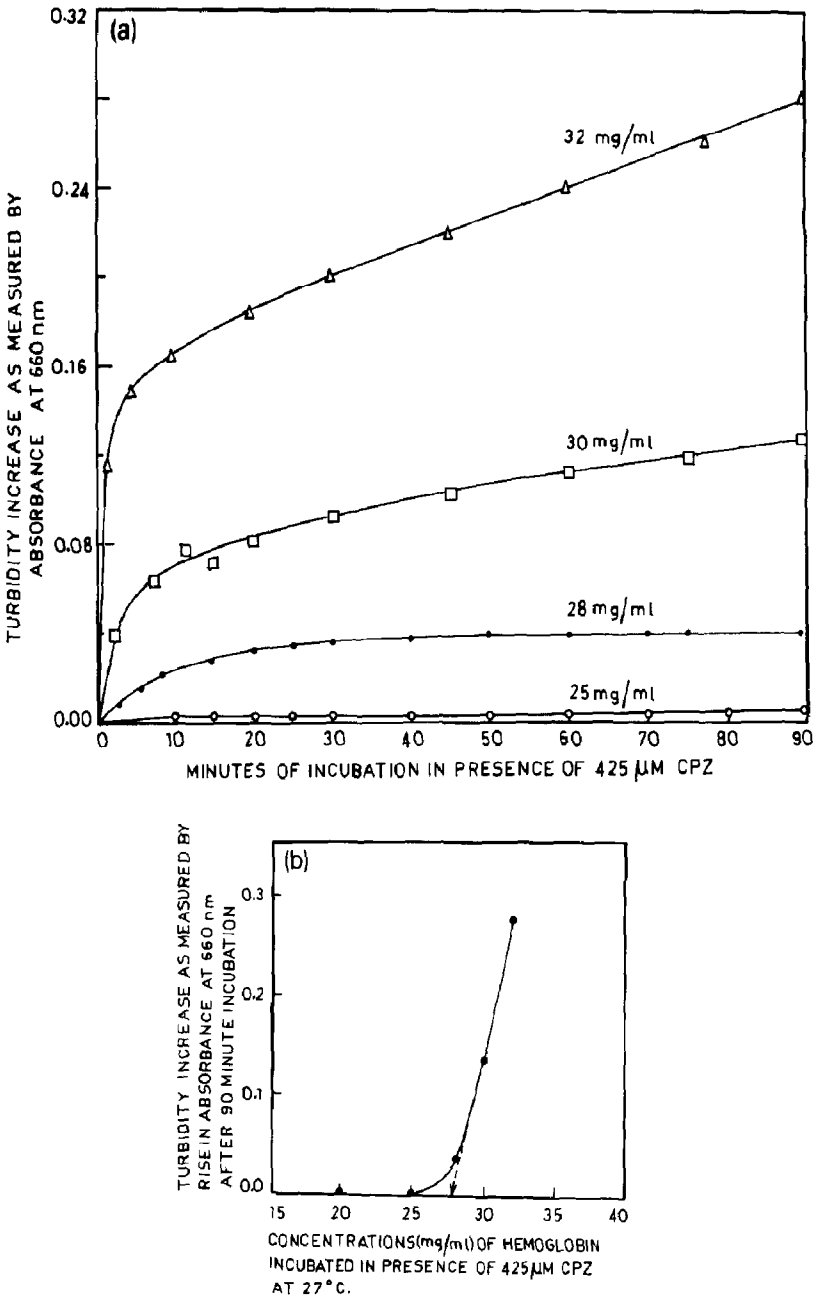

Fig. 1. (a) Time course of the increase in turbidity of different concentrations of hemoglobin (as indicated in the figure) treated with 425 μM CPZ at 27°C. 3 ml of hemoglobin of a particular concentration in 0.15 M PBS (0.15 M NaCl+0.001 M sodium phosphate buffer, pH 6.8) were taken in each of the two quartz cuvettes. To the first cuvette, CPZ was added to a final concentration of 425 μM. The absorbance of the sample in the first cuvette at different time with reference to the second cuvette containing the respective concentration of untreated hemoglobin were monitored at 660 nm in Carl Zeiss PMQ II Spectrophotometer. (b) Typical estimation of critical aggregation concentration (arrow marked) of hemoglobin corresponding to 425 μM CPZ from the plot of increase in turbidity of CPZ-treated hemoglobin (90 min incubation at 27°C) as a function of hemoglobin concentration (mg/ml). Extrapolation of the plot on the abscissa gives the estimate of critical aggregation concentration.

the physiological importance of such binding studies.

However, in all of our previous work hemoglobin concentration not greater than 10–12 mg/ml was used. On the other hand, the intracellular concentration of hemoglobin within normal human RBC is about 300 mg/ml [9]. This fact urged us to extend our studies on the effect of CPZ on normal hemoglobin of concentration higher than that we previously worked with.

The present report is our novel observation that normal human hemoglobin exceeding a certain minimum concentration (called critical aggregation concentration), undergoes aggregation in presence of CPZ; and the critical aggregation concentration of hemoglobin decreases with the increase of CPZ concentration. Observation of CPZ-treated hemoglobin under the electron microscope clearly reveals that the drug-treated normal hemoglobin aggregates in filament form, the average width of the filament being nearly equal to that of the untreated hemoglobin molecule. Possible modes of such filamentous aggregation has been discussed.

2. Materials and methods

Normal hemoglobin from the blood donated by healthy human volunteers (neither having any previous record of genetic disease arising from abnormality of blood nor having any drug addiction) was isolated and characterised according to the method described elsewhere [7,8]. Red blood cells (RBC) of those volunteers showed normal cell envelope under microscope. No sickle cell or any abnormally shaped cells were observed in the RBC donated by the volunteers. Isolated hemoglobin was almost in 100% oxygenated form [8] and of molecular weight 66,800 (tetrameric). Electron micrograph of the isolated hemoglobin (see Fig. 2a in Section 3) reveals its average width to be 65 ± 10 Å (S.D.) and 72 ± 8 Å (S.D.) along two mutual perpendicular directions respectively (S.D. is the abbreviation for standard deviation).

Molar concentrations of hemoglobin and CPZ (chlorpromazine hydrochloride, obtained as a gift from Sun Pharmaceuticals, India) were determined from their peak absorbances at 415 nm ($\epsilon = 125$ mM^{-1} cm^{-1}) and at 305 nm ($\epsilon = 4000$ M^{-1} cm^{-1}) respectively. Aggregation of the drug-treated hemoglobin was monitored by observing the increase of absorbance of the treated hemoglobin at 660 nm or at 720 nm with reference to untreated ones. Neither CPZ nor hemoglobin alone has any significant absorbance at the above two wavelengths. So the increase in absorbance of CPZ-treated hemoglobin at 660 nm as shown typically in Figs. 1a and b can be ascribed to the increased scattering or turbidity due to gradual aggregation of hemoglobin at different times of incubation in the presence of CPZ.

3. Results

Hemoglobin at 25 mg/ml concentration practically shows no absorption increase in presence of 425 μM CPZ (Fig. 1a). When hemoglobin exceeds a concentration value of 28 mg/ml (later on characterised as the critical aggregation concentration; Fig. 1b), a sharp increase in absorption of the CPZ-treated hemoglobin occurs within about 2 minutes of incubation followed by a relatively slow rate of increase. After 90 minutes of incubation at 27°C in the presence of CPZ, hemoglobin of concentration just above this critical value becomes markedly viscous compared to that in absence of CPZ, as observed from the much reduced rate of movement of the CPZ-treated sample through the capillary of an Ostwald viscometer. After 48 hours of incubation in the presence of CPZ at 27°C, aggregation of hemoglobin taken at a concentration much above this critical value, occurs to such an extent that it becomes like a sticky gel and the contents of the tube do not pour down instantaneously upon tilting of the test tube. Figure 1b shows the typical estimation of critical aggregation concentration of normal hemoglobin corresponding to 425 μM CPZ. Critical aggregation concentrations of normal hemoglobin corresponding to various CPZ concentrations ranging between 200 to 600 μM are shown in Table 1. As the concentration of CPZ is increased, the aggregation concentration

of normal hemoglobin decreases indicating that the presence of CPZ favours the aggregation process.

In order to exclude the possibility of the contribution by methemoglobin, if formed at all in presence of CPZ, to the above absorbance increase, absorbance of CPZ-treated hemoglobin was monitored also at 720 nm, which is far away from the wavelength maximum (630 nm) characteristic for methemoglobin [7]. At 720 nm the extinction coefficient value of methemoglobin is practically of little significance. It was found that the absorption increase of the drug-treated hemoglobin occurred also at 720 nm and the time course of the absorption increase at 720 nm (data not shown) was of similar nature as that measured at 660 nm. Critical aggregation concentration of hemoglobin corresponding to some particular CPZ concentration was found to be the same, irrespective of the wavelength at which the

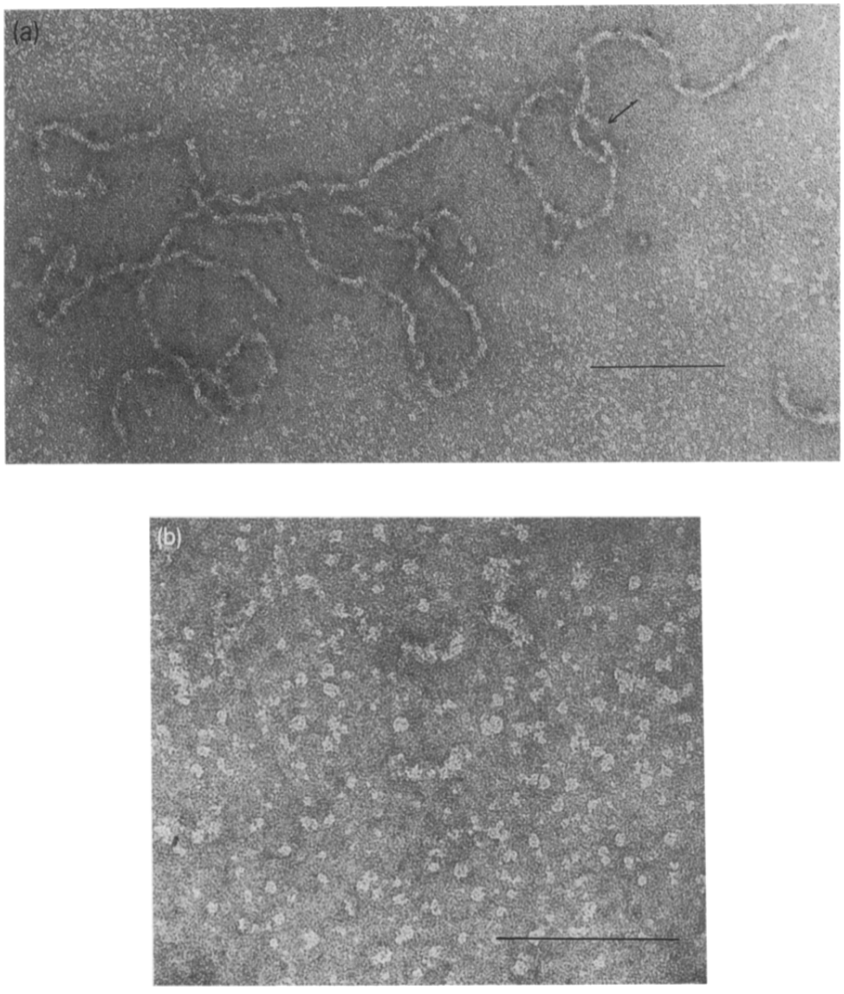

Fig. 2. (a) Electron micrograph of control hemoglobin. Sample of hemoglobin (60 mg/ml) was incubated for 48 hours at 27°C before being stained with 2% uranylacetate and observed under Philips Electron Microscope (Model 420T). The bar on the figure indicates 1000 Å. (b) Electron micrograph of CPZ-treated hemoglobin. Sample of hemoglobin (60 mg/ml) was treated for same time period at 27°C in presence of CPZ (600 μM), and then electromicrographed as above.

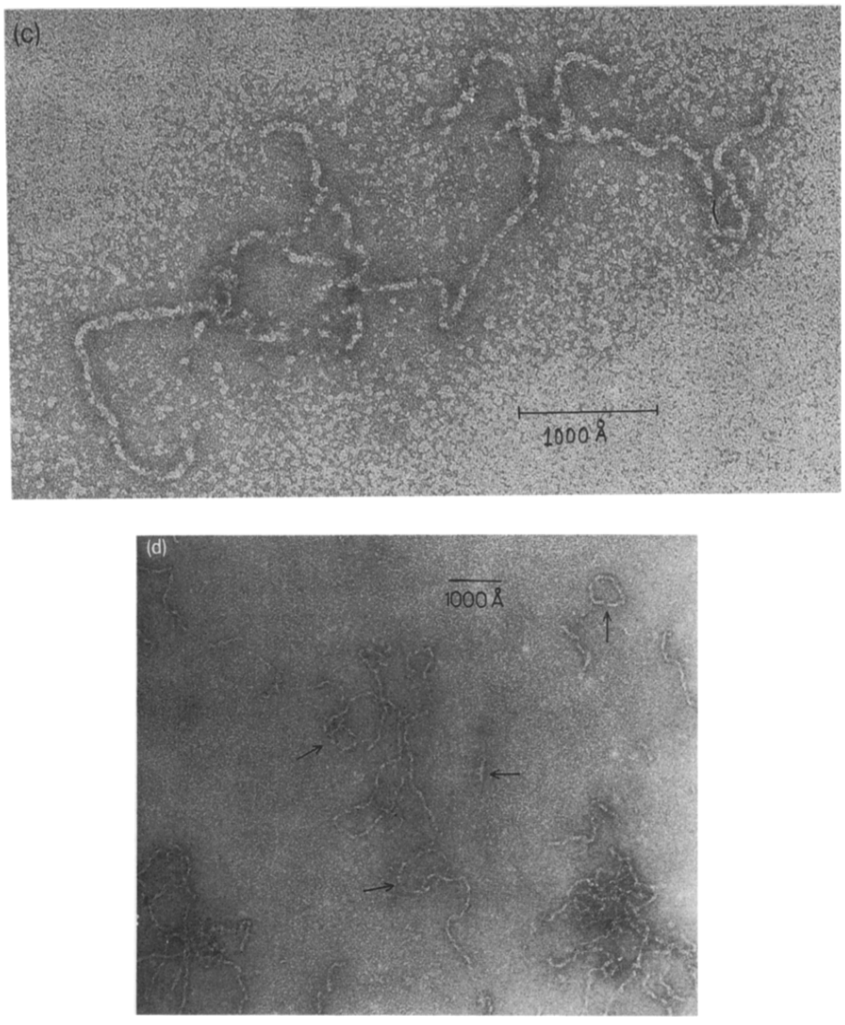

Fig. 2. (c) Electron micrograph of CPZ-treated hemoglobin showing branching and circle formation. (d) Electron micrograph of CPZ-treated hemoglobin at low magnification.

absorption was monitored. It can therefore be concluded that the absorption increase at 660 nm or at 720 nm cannot be ascribed to the formation of methemoglobin.

In order to check the reversibility of the aggregation process, 1 ml of hemoglobin (60 mg/ml) was first incubated with 600 μM CPZ for 48 hours at 27°C to attain sufficient turbidity (as measured by absorbance increase at 660 nm) and gel-like appearance. It was then diluted with 4 ml of PBS buffer so that the final concentration of hemoglobin in the diluted complex falls below the critical aggregation concentration corresponding to 600 μM CPZ. The diluted sample was stirred gently and was left standing overnight at 27°C. It was found that the turbid gel went into the solution. Absorbance of the solution at 660 nm with reference to the similarly diluted and untreated control hemoglobin was observed to be practically zero, indicating the reversibility of the aggregation process.

Tetrameric hemoglobin is slightly oblate shaped with widths 65 Å and 72 Å along its minor and major axis respectively as is evident from

Table 1

Critical aggregation concentration of hemoglobin corresponding to different CPZ concentrations

Concentrations of CPZ (μM)	Critical aggregation concentration of hemoglobin [a] (mg/ml)
220	35
300	33
350	31
425	28
500	27
600	25

[a] Critical aggregation concentration of hemoglobin corresponding to each of the above CPZ concentrations were determined as is shown in Fig. 1b.

electron micrographs (Fig. 2a). Observation of CPZ-treated hemoglobin under electron microscope gives clear evidence of filamentous aggregation of drug-treated hemoglobin (Fig. 2b). The average width of the filament has been measured to be 75 ± 8 Å (S.D.) which is nearly equal to the width of the untreated hemoglobin molecule along its major axis. Figure 2d is the low magnification (magnification × 120,000) electron micrograph providing good indication of the overall population of the aggregates in larger field. Filaments and circles (marked by arrows) are seen to be truly abundant in the micrograph. The start of the branching in a short piece of filament is indicated by arrow. Average dimension of the uranyl acetate stained preparation of hemoglobin tetramer that we have observed under the electron microscope is about 10% greater than the known dimension measured by X-ray diffraction analysis [10]. Mellema et al. [11] in their detailed assessment of the aspects of negative staining in the electron microscopy of several low molecular weight proteins, also observed greater dimensions of the protein molecules as compared to those obtained by X-ray diffraction. They attributed this to certain factors such as orientation of the molecules, thickness of the stain layer, extent of defocussing, etc. Among these factors, orientation of the protein molecule seemed to them of much importance because electron microscopy of negatively stained preparations of ten projections of the model of a papain molecule at different orientations yielded different dimensions, mostly too large.

4. Discussion

Appearance of a lag-time in the time dependent absorbance increase profile of the CPZ-treated hemoglobin sample could not be resolved at CPZ concentrations lying within the range 400–600 μM. However, a small lag-time of about five minutes was in fact observed at lower CPZ concentrations in the range of 200–350 μM.

At higher CPZ concentration, a greater amount of hemoglobin will be bound to the drug than at lower drug concentration. Hence at higher drug concentration a greater number of bound hemoglobin is available for the nucleation step to start with, so that the nucleation rate for aggregation is faster at higher CPZ concentration. Lag-time is therefore not observed at higher CPZ concentration.

It is relevant to mention here that the concentration of CPZ in RBC of patients who were administered 900 mg of CPZ as daily therapeutic dose was found to be 360 μM [6] which falls within the experimental range of CPZ concentrations (200–600 μM) we have used. In the light of our above observation, it is likely to conceive that hemoglobin in RBC (intracellular concentration of hemoglobin being 300 mg/ml) [9] will aggregate to an appreciable extent in the presence of incorporated CPZ used at above-therapeutic doses, causing deleterious effects such as cell rupturing or change in RBC morphology [12] and function. Under the light microscope, we have in fact observed that the RBC cell envelope changes after being treated with CPZ in the above concentration range (Fig. 3) and oxygen is released from such treated suspensions of RBC (Fig. 4).

Occurrence of a critical concentration in this aggregation process suggests that the aggregation of drug-treated hemoglobin is possibly initiated by a nucleation step requiring a certain minimum concentration of drug-bound hemoglobin followed by a subsequent propagation step. Such a mechanism for the aggregation process has already been formulated as the linear aggregation

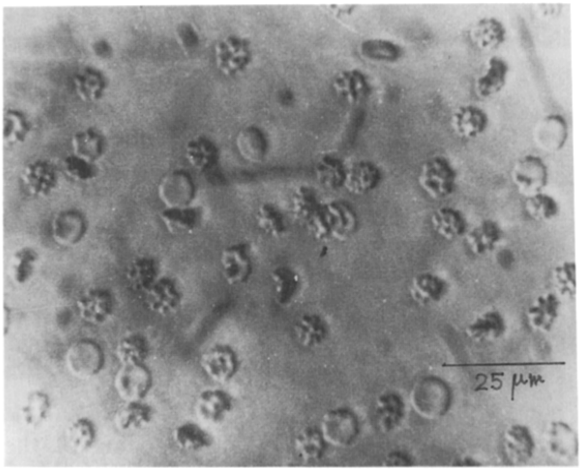

Fig. 3. Photomicrograph of a CPZ-treated human red blood cell. A suspension of red blood cell (2×10^7 RBC/ml) in PBS buffer, pH 6.8 was treated with 400 μM CPZ for 20 minutes at 27°C and then photomicrographed at a final magnification of 800.

theory [13,14] and is found to occur in many cases, like the polymerisation of G-actin to F-actin [15], formation of polysheath protein P18 from its monomeric unit [16], etc. Following the above theory [14], the association constant of aggregation of drug-bound hemoglobin measured as the inverse of the critical concentration was found to vary from 3×10^3 M^{-1} to 4×10^3 M^{-1} corresponding to the concentrations of CPZ we have used.

In studies on the assembly of tubulin, Na and Timasheff [17,18] observed aggregation effect in presence of anticancer drug vinblastine. They referred it as Cann and Goad type interaction [19] in which the ligand induced conformational changes of the protein might be a responsible factor for such aggregation. Phenothiazine drug, CPZ, with its positively charged tail region binds with the hemoglobin, while its bulky non-polar ring portion with a bend about its north–south axis is exposed to the polar aqueous environment ([8], Ph.D. Thesis of M. Bhattacharyya, 1991). We have also reported earlier that upon binding, CPZ induces a change in conformation of hemoglobin [8] with the concomitant release of oxygen. The fluorescence spectra of CPZ-treated hemoglobin [8] reveals clearly that the conformation change of the drug-treated hemoglobin occurs in such a way that the non-polar tryptophans of hemoglobin are exposed to the more polar environment. The tryptophans of the drug-bound hemoglobin along with the non-polar bulky ring portion of the bound drug endow the hemoglobin a strong aggregation tendency so that the contact with the polar environment is minimum. Such

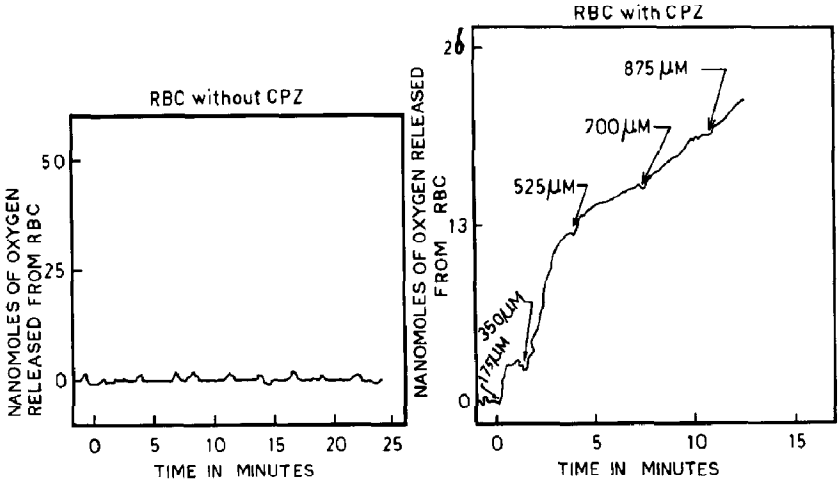

Fig. 4. Release of oxygen from CPZ-treated red blood cell. Oxygen release has been monitored by Gilson 5/6 oxygraph machine. 2 ml suspension of red blood cells (2×10^7 cells/ml) in PBS buffer, pH 6.8 was put in the stoppered cell of the Gilson 5/6 oxygraph machine. Nanomoles of oxygen released were registered after addition of CPZ of various final concentration (arrow marked) to the RBC suspension at 27°C.

tendency to aggregate might ultimately lead to filamentation of drug-treated hemoglobin.

CPZ binding to hemoglobin will lead to conformation change so that, if at least, one non-polar tryptophan residue gets exposed to the polar environment, then the association tendency between the bulky non-polar ring portion of CPZ and the exposed tryptophan in the neighbouring hemoglobin might lead to formation of linear filament. This has been denoted mode A type association in Fig. 5. It could have been also the other mode, mode B, where tryptophan–tryptophan and drug–drug association between two neighbouring drug-bound hemoglobin molecules are simultaneously active. Since drug–drug association is not very significant in the above range of CPZ concentration we have studied [8], it is most likely that the mode A type association is operative in such process. Moreover, studies on fluorescence quenching of free tryptophans in presence of CPZ [8] indicated clearly that the tryptophans interact with CPZ with an association constant of the order of $3 \times 10^3\ M^{-1}$, which is in close agreement with the association constant for aggregation of drug-bound hemoglobin. This further supports the notion of CPZ–tryptophan interaction in a mode A type association.

If, on the other hand, more than one drug is bound to hemoglobin, or more than one tryptophan residue is exposed to a polar environment, then following the mode A type of association there will be a possibility of branching of the filament or formation of a circle as shown in Fig. 5. Such branching and circle formation is evident from the electron micrograph of CPZ-treated hemoglobin (Fig. 2C).

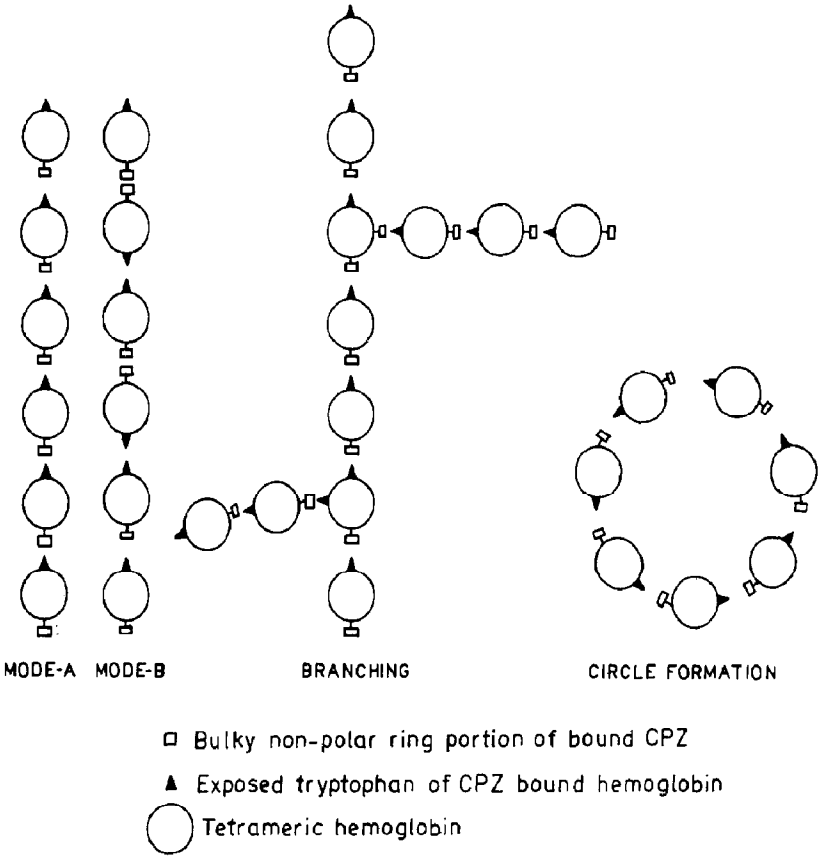

Fig. 5. Different modes of association of CPZ-treated hemoglobin. Mode A indicates CPZ–tryptophan association between two neighbouring hemoglobin molecules, while mode B indicates tryptophan–tryptophan and drug–drug association.

Acknowledgements

This work has been done with the financial assistance from Council of Scientific and Industrial Research (CSIR), Government of India. We are grateful to Dr. S.C. Pal, Director, National Institute of Cholera and Enteric Diseases, Calcutta for his kind permission to use the electron microscope facility. Part of this work is an excerpt from the Ph.D.(Sc.) thesis of M. Bhattacharyya, University of Calcutta, 1991.

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