RAPID BINDING OF PMB TO NON-SH GROUPS ON HEMERYTHRIN[+]

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SUMMARY

Using the flow dialysis technique we find that p-mercuribenzoate binds to the non-heme iron protein hemerythrin at a number of sites other than the sulfhydryl group. This non-sulfhydryl binding probably occurs faster than formation of the sulfur-mercury bond.

The reactivity of the cysteinyl side chain in proteins has been studied often in order to obtain information about protein conformation. Because of its apparent specificity, and ease of experimental manipulation, p-mercuribenzoate (PMB) has been utilized to probe for conformational changes, (e g.; 1,2) assuming that reaction occurs only at sulfhydryl (SH) groups. Binding of excess organic mercurials at non-SH sites has been previously reported, however (3,4,5). PMB binding to non-SH sites could possibly interfere in the studies of protein sulfhydryl reactivities provided non-SH binding occurred at rates comparable to the formation of the sulfur-mercury bonds. While studying the sulfhydryl reactivity of hemerythrin, an octameric, non-heme iron containing protein from sipunculids (6,7), we observed kinetic irregularities which we postulated were due to PMB binding at non-SH sites.

To determine whether PMB binds rapidly to hemerythrin at sites other than the single SH of each subunit we turned to the technique of flow dialysis recently described by Colowick and Womack (8). The basis of their

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method is that the rate of ligand diffusion across a dialysis membrane is directly proportional to the concentration of the non-complexed ligand. This method is more powerful than other binding procedures due to the very short time in which ligand binding may be measured. Using a flow dialysis cell of our own construction we have been able to show that PMB reacts at a number of non-SH sites more rapidly than at the cysteinyl side chain.

METHODS

Methemerythrin was prepared from the coelomic fluid of Goldingia gouldii (Marine Biological Laboratories, Woods Hole, Mass.) by the procedure of Klapper and Klotz (9). The sulfhydryl content of the stock methemerythrin solutions was determined by PMB titration (10). The kinetics of the PMB reaction with protein SH groups was followed at 250 mμ using a Cary 16 spectrophotometer (Cary Instruments,

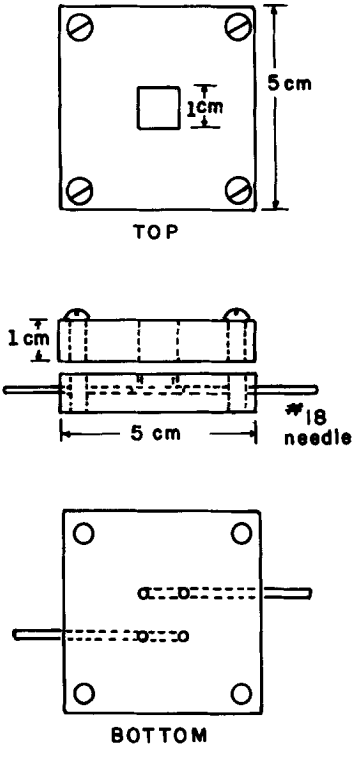

Figure 1 A schematic diagram of flow dialysis cell

Monrovia, Calif.). Binding experiments were performed using the flow dialysis cell schematically diagrammed in figure 1. The cell was designed with no lower chamber in order to minimize dead volume. Buffer was pumped underneath the dialysis membrane through entrance and exit holes drilled into the surface of a plexiglass block. The membrane was manually stretched and laid over the holes. The top half of the cell, a plexiglass block with a square hole cut into the center, was screwed onto the bottom plate with a piece of parafilm acting as a gasket. To start the experiment buffer is pumped beneath the membrane with a positive displacement pump obtained from Technicon Corporation (Tarrytown, N.Y.). The cyclic pressure exerted by the pump causes the membrane to rise and fall mixing the solution beneath. The effluent is directed through a quartz flow cell (Precision Cells, New York, N.Y.), and the solution absorbance measured at 232 mμ. After buffer, or buffer and protein is placed into the upper half of the cell, and a stable baseline achieved, PMB is added. Under the conditions of our experiment, at a buffer flow rate of 0.67 ml/min steady state was achieved in 2.5-3.0 minutes. The solution was thermostated by placing the entire cell in an insulated box which was cooled by nitrogen gas previously passed through a liquid nitrogen bath. The temperature was regulated by adjusting the gas flow.

RESULTS AND DISCUSSION

We have previously determined that the absorbance change accompanying PMB titration of the methemerythrin SH groups is an accurate measure of sulfur-mercury bond formation, and does not reflect PMB binding to non-SH sites, or protein conformational alterations (11). Thus, $\Delta\epsilon_{250}$ measured for the PMB reaction with methemerythrin is $7.99 \pm 0.38 \times 10^3\ M^{-1}cm^{-1}$, and ϵ_{250} for PMB mixed in excess with fully reacted methemerythrin is $4.97 \pm 0.29 \times 10^3\ M^{-1}cm^{-1}$. This compares well with $\Delta\epsilon_{250}$ reported by Boyer (10) for the reaction of PMB with cysteine

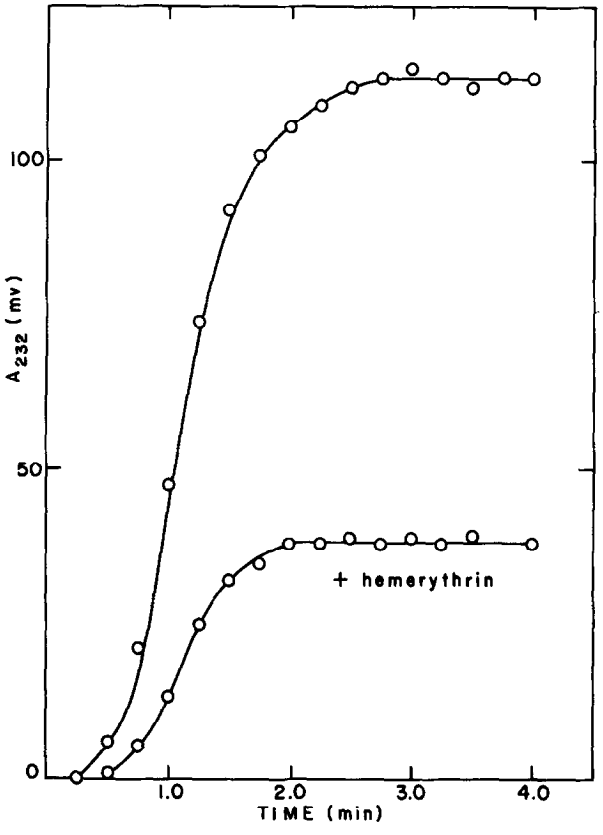

Figure 2 PMB binding to methemerythrin
PMB was added to the dialysis cell at time zero using the experimental procedure described in the text. PMB and protein SH concentration were 3.71×10^{-4} M and 2.54×10^{-4} M respectively. The experiment was performed at 10 ± 1 °C in 0.05 M Tris (cacodylate), pH 7.2, 0.2 M NaF.

7.5×10^{3} $M^{-1}cm^{-1}$, and with the extinction coefficient of PMB in the absence of protein, $\epsilon_{250} = 4.86 \pm 0.01 \times 10^{3}$ $M^{-1}cm^{-1}$. In addition all SH groups react with PMB. By PMB titration the ratio of protein iron to SH was 1.80 ± 0.11 (11), as compared to the 2 to 1 ratio determined from amino acid analysis (12).

Using the flow dialysis cell described in the methods section we were able to measure the amount of protein bound PMB within three minutes after mixing. Addition of PMB was followed by a lag due to dead space in the flow system. At approximately 0.5 minutes the absorbance increased and then reached a plateau 2 to 2.5 minutes after mixing (Fig.2). In the

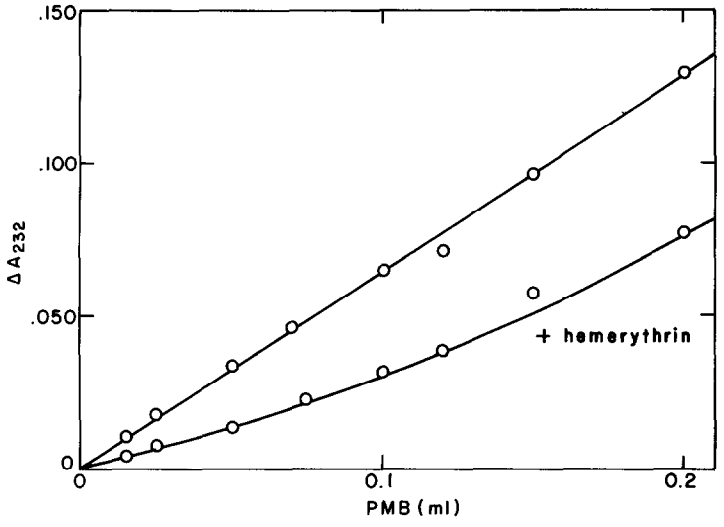

Figure 3 PMB binding to methemerythrin at the steady state Plateau absorbance at three minutes is plotted against the initial amount of PMB added: PMB stock, 3.71×10^{-3} M: Total volume 0.35 ml. Other conditions are described in the legend of figure 2.

presence of methemerythrin the plateau absorbance is lower than in the absence of the protein due to binding of the organic mercurial by methemerythrin. When only PMB is added to the upper cell, the plateau absorbance of the effluent stream increases linearly with PMB concentration (Fig. 3). Hence, the absorbance of PMB in the effluent stream is a measure of free organic mercurial. With protein present the free PMB concentration is markedly decreased (Fig. 3). From the curves of figure 3 the amounts of protein bound PMB at various initial mercurial concentrations were calculated and are shown as the top curve of figure 4. Clearly, more PMB binds than there is SH available for reaction.

Because there is a plateau after 2.5 minutes, all, or almost all of the bound PMB must react within 2.5 minutes. We independently measured the reaction of PMB with the methemerythrin SH groups under the same experimental conditions. The lower curve in figure 4 shows the amount of SH reacted in the first three minutes after mixing. Within this time

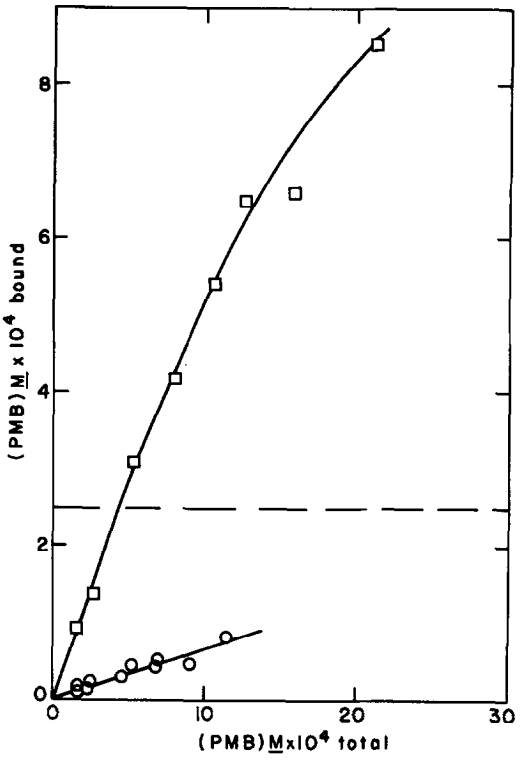

Figure 4 Comparison of PMB binding determined by flow dialysis, and by absorbance change at 250 mμ
Upper curve: Total PMB bound to hemerythrin. Lower curve: Amount of PMB bound to SH within three minutes as determined by absorbance changes at 250 mμ. Dashed line: Total SH concentration. Experimental conditions were those described in figures 2 and 3.

the SH groups remain largely unreacted. Further, only a small fraction of protein bound PMB, as measured by dialysis, is attached to the protein sulfhydryl. Because the major part of the PMB attached to the protein reached an apparent equilibrium within three minutes, while the reaction at the SH was still unfinished, we conclude that PMB binds to methemerythrin more rapidly at non-SH sites than it reacts with the SH groups.

Assuming that a large percentage of the bound mercurial is not attached to the protein sulfhydryl, we determined the apparent binding constant for the reaction at the non-SH sites. The data were analyzed by the graphical procedure of Jenkins and Taylor (13). The graphical plot

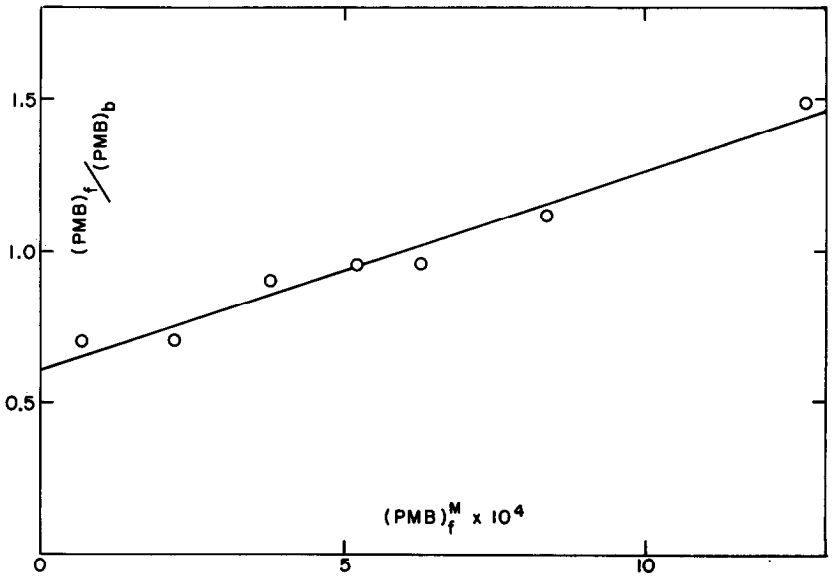

Figure 5 Binding affinity of PMB for non-SH sites of methemerythrin The data of figure 4, upper curve plotted by the graphical method of Jenkins and Taylor (13). The subscripts f and b represent free and bound PMB.

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is linear (Fig. 5) indicating that the PMB molecules bind to the non-sulfhydryl sites with the same intrinsic binding constant. Over the protein concentration range 2.17 - 3.56 x 10^{-4} M (expressed as monomer concentration) the apparent number of binding sites was calculated as 5.7 $\pm$ 0.3 per protein subunit, and the binding constant obtained was 1.23 $\pm$ 0.31 x 10^3 M. Since PMB binds stoichiometrically with the SH, and since the binding curve obtained in the dialysis experiment is hyperbolic, the protein bound PMB as determined by dialysis is due almost entirely to non-SH complexed mercurial. This is reasonable due to the lag introduced by the dead space in the flow system. The PMB measured as bound at three minutes was attached to the protein at an earlier time, most likely within the first minute after mixing.

PMB binds extensively to methemerythrin at sites other than the SH groups, though at present we do not know the chemical nature of these sites. Non-sulfhydryl binding does not interfere with the analytical determination

of sulfhydryl groups in the protein, due most likely to the lower affinity of PMB for the non-SH sites. However, this rapid binding of PMB may result in complications when studying the kinetics of sulfur-mercury bond formation. Any conclusions based on the kinetics of the PMB-SH reaction of proteins must be treated cautiously until the effects of the possible secondary binding are taken into account.

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