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CHANGES IN CONFORMATION AND FUNCTION OF HEMOGLOBIN AND MYOGLOBIN INDUCED BY ADSORPTION TO SILICA

Ben E. Hallaway, Philip E. Hallaway, William A. Tisel, and Andreas Rosenberg

Department of Laboratory Medicine and Pathology
University of Minnesota, Minneapolis, Minnesota 55455

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SUMMARY

Adsorption of myoglobin (Mb) or hemoglobin (Hb) to silica (Cab-O-Sil) causes marked alterations in protein hydrogen exchange kinetics. The exchange is slower for cyanometMb and faster for both cyanometHb and oxyHb in adsorbed state than for the corresponding species in the free state. For Hb, adsorption increases oxygen affinity (P_{50} = 12.9 mmHg vs. 16.7 for free) and decreases cooperativity (n = 2.05 vs. 2.87 for free). Myoglobin has the same oxygen affinity in both the free and adsorbed states.

INTRODUCTION

Although there is considerable literature on the interaction of proteins with surfaces, knowledge about dynamic conformational and functional changes resulting from surface adsorption is meager. Knowledge of such changes is important to the application of synthetic devices for the cardiovascular system. An infrared spectroscopic study has shown that serum albumin, prothrombin, and fibrinogen are strongly bound to a silica surface (Cab-O-Sil), but that changes in protein conformation, as seen by this technique, appeared to be small or nonexistent (1). On the other hand, the same technique supplemented by ellipsometry indicated that gamma globulin adsorbed to silica underwent a shape change which was dependent on surface concentration (2).

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We have chosen Cab-O-Sil as an experimental hydrophilic surface to study the effect of adsorption on the structure and function of hemoglobin and myoglobin. These are well characterized proteins whose functional properties are sensitive to their environment.

MATERIALS AND METHODS

Human hemoglobin was prepared from outdated red cells as described by Barksdale et al. (3). Whale metmyoglobin, obtained from Seravac Laboratories and Sigma Chemical Co., was converted to cyanometMb without further purification for hydrogen exchange studies and reduced to ferroMb by the method A. Hayashi et al. (4) for oxygen binding studies.

Hydrogen exchange was carried out as described previously (5) except as noted below. Myoglobin, as cyanometMb, was equilibrated with tritiated water while hemoglobin was tritiated in the oxyHb state and converted to cyanometHb prior to initiation of out-exchange. Hydrogen exchange in the presence of Cab-O-Sil was carried out as follows: A few milligrams of Cab-O-Sil was added to a number of 1.5 ml polypropylene centrifuge tubes. Solvent tritium was removed from the protein sample by chromotography at 5^{o} on a Sephadex G-25 column equilibrated with sodium phosphate buffer (pH 7.0, I = 0.2) thereby initiating out-exchange. A volume of 0.5 ml of the eluate was quickly transferred to each of the tubes, which were then incubated in a 5^{o} water bath. The Hb concentration after the first filtration was about 0.5 mM in tetramer, high enough to keep dimer concentration insignificant. At intervals, the solution above the Cab-O-Sil was filtered on a second small Sephadex G-25 column for determination of the hydrogens remaining unexchanged in the free protein. The adsorbed protein was washed free of unbound protein by resuspending in 1 ml of ice-cold sodium phosphate buffer (pH 6.0, I = 0.2) and discarding the supernatant after centrifugation for 10 seconds at 12,000 x g. This washing was done twice. The washing steps were carried

out at pH 6.0 and 0^{o} to minimize further out-exchange during handling. Then 1 ml of Drabkin's reagent containing 0.1 M sodium dodecyl sulfate was added to release the protein from the adsorbent. After centrifugation an aliquot of this supernatant was scanned in the Soret region and an aliquot counted by liquid scintillation. Quantitation of concentration and tritium activity in the Cab-O-Sil free phase from the second separations on G-25 was carried out exactly as for the adsorbed protein. Calculation of the number of hydrogens remaining unexchanged per subunit was done by standard methods.

Hb oxygen binding was measured at 37^{o} in 0.1 M potassium phosphate (pH 7.0) with a Hem-O-Scan Oxygen Dissociation Analyzer (American Instrument Co.). For determinations in the bound state, Cab-O-Sil was saturated with Hb, washed two times with 0.1 M potassium phosphate (pH 7.0), and the pellet introduced into the Hem-O-Scan. Oxygen binding of Mb was carried out in 0.05 M Bis-Tris, 0.1 M NaCl (pH 7.2) at 37^{o}.

RESULTS

As shown in Fig. 1, the hydrogen exchange rate of cyanometHb was greatly increased when adsorbed to Cab-O-Sil. Adsorbed oxyHb also showed an increased exchange rate when adsorbed to Cab-O-Sil, but this increase was smaller than that seen for adsorbed cyanometHb, e.g. at 100 minutes oxyHb had a difference of six hydrogens remaining unexchanged between free and bound forms while cyanometHb showed a difference of 18. The exchange rates of cyanometHb, oxyHb and cyanometMb molecules that remain in solution in the presence of Cab-O-Sil are the same as in the absence of Cab-O-Sil. These exchange rates agreed well with previously published values (5,6). In contrast to Hb, cyanometMb had a much slower exchange rate in the adsorbed state than in the free state. The liganded forms of Hb and Mb in solution had very similar exchange rates.

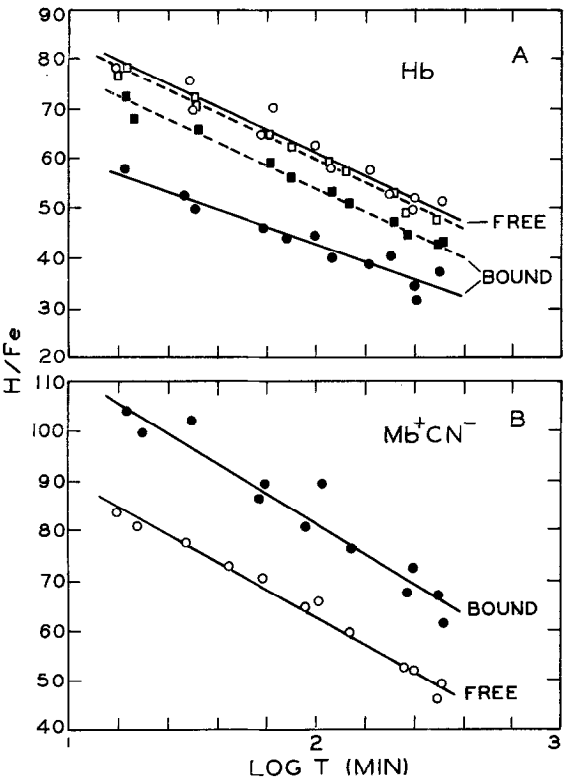

Figure 1. Comparison of hydrogen exchange kinetics for free and Cab-O-Sil bound proteins in sodium phosphate buffer (pH 7.0, I = 0.2) at 5°C. A, oxyHb ---- free (□) and bound (■); cyanometHb —— free (○) and bound (●). B, cyanometMb —— free (○) and bound (●). These data points represent two separate determinations for each species. The straight lines drawn through the points were fitted by least squares.

A comparison of oxygen binding by hemoglobin in the free and adsorbed states is presented as Hill plots in Fig. 2. Adsorption to Cab-O-Sil increased Hb oxygen affinity considerably. The value of P_{50} decreased 22% (16.7 for free and 12.9 mmHg for bound) and cooperativity decreased 31% (n = 2.87 for free and 2.05 for bound) upon adsorption of Hb. Adsorption to Cab-O-Sil did not affect Mb oxygen binding properties. The oxygen binding isotherms of the fraction of Hb and Mb that remain in solution in the presence of Cab-O-Sil are unaltered.

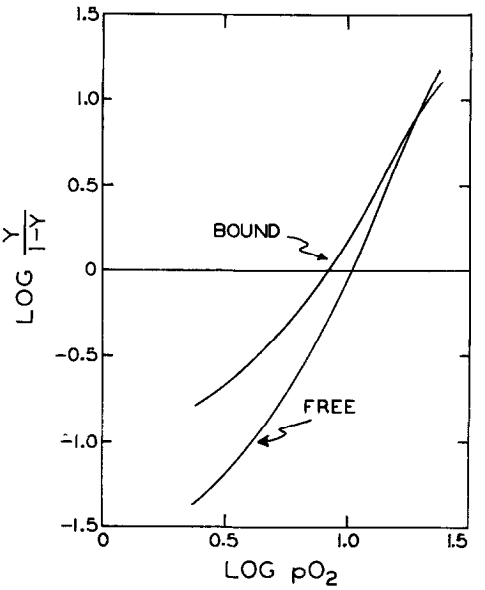

Figure 2. Hill plots comparing oxygen binding properties of Hb bound to Cab-O-Sil and in free solution in 0.1 M potassium phosphate (pH 7.0) at 37°C.

Titration of a weighed amount of Cab-O-Sil in sodium phosphate buffer (pH 7.4, I = 0.2) at 5°C with increments of oxyHb while following the absorbance of the supernatant gave a binding value of 4.4×10^{-6} mmoles heme/mg silica. Direct measurement of the amount of oxyHb bound to Cab-O-Sil at saturating Hb concentrations gave a value of 6.9×10^{-6} mmoles/mg. For cyanometMb under the same conditions, binding values were 2.5×10^{-6} mmoles by titration and 4.1×10^{-6} mmoles/mg by direct measurement. By both techniques the amount of hemoglobin bound was 1.7 times greater than for myoglobin.

DISCUSSION

Adsorption of cyanometHb and oxyHb to Cab-O-Sil leads to an increase in the hydrogen exchange rates of these molecules as compared to the rates of the nonadsorbed proteins. The adsorption of cyanometMb, on the contrary, leads to a decrease in the exchange rate compared to

cyanometMb in solution. Hydrogen exchange kinetics express the dynamic aspects of protein structure, i.e., fluctuations of the conformation leading to increased accessibility to solvent result in an increase in exchange rates (7,8). In the case of hemoglobin, the change in oxygen binding isotherms upon adsorption to Cab-O-Sil (decreased cooperativity and increased affinity) could be explained most easily by an increase of the fraction of molecules present in the form of dimers, because Hb dimers exhibit higher oxygen affinity and lower cooperativity than the tetramers. This would also explain the observed changes in hydrogen exchange, since dimers of liganded ferro- and ferrihemoglobin show increased exchange rates compared to tetramers.[1] The difference in adsorption induced changes for ferro and ferri forms would in such a case be a result of differences in the tetramer-dimer dissociation constant. Unfortunately, it is at present difficult to establish from published values if the tetramer-dimer dissociation constant for liganded ferro and ferri forms differs and if so, what order of magnitude this difference represents. Consequently, we have at present no direct evidence that the tetramer-dimer equilibrium of adsorbed hemoglobin is shifted towards dimer. It seems equally probable that a multisubunit protein such as hemoglobin is under considerable structural strain when adsorbed to a surface by multiple binding sites, and that such a strain is sufficient to alter the dynamics of protein conformation, favoring the more solvent accessible forms.

Myoglobin, a single chain protein, does not show an increase in accessibility to solvent. On the contrary, it shows a decrease in hydrogen exchange rate on adsorption. This could be due to two obvious causes. First, it could be due to a loss of interface between water and protein due to contact with the solid surface. This would require a relatively large decrease in surface area of Mb in contact with solvent,

[1] A.D. Barksdale, unpublished observations.

an amount which seems unlikely. For this reason, we favor the second possibility, that of increased structural stability. Such an increase in stability would not be expected to alter the oxygen binding parameters of Mb which normally shows no cooperativity in oxygen binding. That no alteration of Mb oxygen binding properties occurs, provides good evidence that adsorbed Mb, like Hb, is still a functionally active molecule.

Although less likely, the possibility that adsorbed Mb exchange behavior is due, at least in part, to a reduction of the area of the protein-water interface cannot be ruled out by experiments described here. Clearly, however, in the case of Hb, the binding data suggest, assuming a monolayer, that only two subunits are in close contact with the silica. This would cause only a relatively small fraction of the surface area of the Hb molecule to be removed from contact with solvent upon adsorption.

We conclude that conformational changes in Hb and Mb result from their adsorption to a hydrophilic silica surface such as Cab-O-Sil, and these changes can be detected by hydrogen exchange kinetics. Hemoglobin in both cyanomet- and oxy- states become more accessible to solvent and cyanometMb becomes less. The increase in oxygen affinity and decrease in cooperativity for Hb upon adsorption is consistent with the view that adsorption causes strain on the Hb molecule leading to decreased subunit "communication". Finally, these results indicate that although Hb and Mb conformations are altered by adsorption to Cab-O-Sil, the proteins are still functional.

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REFERENCES

1. Morrissey, B.W. and Stromberg, R.R. (1974) J. Colloid Interface Sci. 46, 152-164.
2. Morrissey, B.W. and Fenstermaker, C.A. (1976) Trans. Am. Soc. Artif. Int. Organs 22, 278-284.
3. Barksdale, A.D., Hedlund, B.E., Hallaway, B.E., Benson, E.S. and Rosenberg, A. (1975) Biochemistry 14, 2695-2699.
4. Hayashi, A., Suzuki, T., and Shin, M. (1976) Biochim. Biophys. Acta 310, 309-316.
5. Hedlund, B.E., Hallaway, P.E., Hallaway, B.E., Benson, E.S., and Rosenberg, A. (1978) J. Biol. Chem. 253, 3702-3707.
6. Benson, E.S., Rossi-Fanelli, M.R., Giacometti, G.M., Rosenberg, A., and Antonini, E. (1973) Biochemistry 12, 2699-2706.
7. Woodward, C.K., Ellis, L.M., and Rosenberg, A. (1975) J. Biol. Chem. 250, 432-439.
8. Ellis, L.M., Bloomfield, V.A., and Woodward, C.K. (1975) Biochemistry 14, 3413-3419.