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CHANGES IN PROTEIN TOPOGRAPHY UPON OXYGENATION

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Communicated by Theodore Shedlovsky, June 11, 1957

The site of attachment of oxygen in the oxygen-carrying pigments has long been specifically identified as the metal in these proteins, the metal being attached to porphyrin groups in hemoglobin and chlorocruorin or directly to side chains of certain amino acid residues in hemerythrin and hemocyanin. It seemed of interest to inquire further whether the bulk of the macromolecule participates in the process of oxygen uptake. Recent work[1–6] has indicated that changes in optical rotation of proteins reflect modifications in over-all configuration. Measurements of specific rotation would thus seem in order.

A few measurements for hemoglobin are available in the older literature.[7, 8] The significance of the values observed [$\alpha^{20°}_{668\ m\mu} \simeq +15°$] is complicated by the presence of the porphyrin prosthetic group and of co-operative interactions between sites. Consequently it seemed appropriate to consider first the behavior of a pigment in which these complications do not occur. An examination was therefore made of the variations in optical rotation upon deoxygenation and oxygenation of hemocyanin, in which oxygen is held by copper attached directly to the protein fabric. In samples of this protein obtained from *Busycon*, the active sites act

independently of each other in isoionic solutions; the oxygenation curve fits a Hill equation[9, 10] with $n = 1$.

Since solutions of hemocyanin are deeply colored, measurements of optical rotation were made with a Bellingham and Stanley polarimeter to which a photomultiplier detector had been attached. Furthermore, in view of the strong absorption of yellow light by this protein, a mercury-cadmium lamp, rather than a sodium lamp, was used, with a strong line at 4358 Å, near the minimum in the absorption curve of the protein.

Some observations are summarized in Table 1. Oxyhemocyanin from *Busycon canaliculatum* showed some small variations in specific rotation ($-56°$ to $-62°$) from sample to sample. A sample with $\alpha = -61.8°$ was treated with $NaHSO_3$, in an amount approximately five times the copper content of the protein solution. Within 15 minutes α dropped to $-34.5°$. Concurrently the blue color, and the associated absorption peak at 580 mμ, disappeared. Likewise, the much more intense absorption band of the oxygenated protein at 340 mμ vanished completely. When the solution was then allowed to stand open to air, the characteristic visible blue and the near-ultraviolet peak at 340 mμ of the oxygenated protein returned, though not to its full intensity, and α rose again to $-59.2°$.

Similar results were obtained with less $NaHSO_3$, in an amount about twice the copper content (Table 1).

TABLE 1

SPECIFIC ROTATIONS OF DEOXYGENATED HEMOCYANIN AND OXYHEMOCYANIN AT 25°C.

	Solution	Specific Rotation, α
1.	Oxyhemocyanin; dialyzed sera (approx. 1 per cent protein)	$-59.0°$, $-56.2°$, $-57.7°$, $-61.8°$
2.	Deoxygenated hemocyanin; Solution 1 + $NaHSO_3$; $NaHSO_3/Cu \simeq 5$	$-34.5°$
3.	Solution 2, exposed to air for 40 hours	$-59.2°$
4.	Oxyhemocyanin	$-62.3°$
5.	Deoxygenated hemocyanin; Solution 4 + $NaHSO_3$; $NaHSO_3/Cu \simeq 2$	$-37.7°$
6.	Oxyhemocyanin	$-59.5°$
7.	Deoxygenated hemocyanin; Solution 6 + glucose oxidase-catalase (0.003 per cent) and glucose (0.018 per cent)	$-21.8°$

As a further check, it seemed desirable to avoid a method of abstracting oxygen from the protein by reduction and to use a method of removal which depleted the dissolved oxygen in solution in equilibrium with the hemocyanin. Despite intensive efforts, complete displacement of dissolved oxygen by means of bubbled high-purity nitrogen could not be attained; although the weak 580 mμ peak of hemocyanin disappeared, it did not prove possible to reduce the intense 340 mμ absorption to the background value. An enzymatic method[11] was therefore used. A commercial preparation of glucose oxidase and catalase at a concentration of 0.003 per cent in the presence of 0.02 per cent glucose completely removed the oxygen from solution, as judged by the disappearance of the 340 mμ band of hemocyanin. As in the bisulfite treatments, this deoxygenated hemocyanin showed a pronounced drop in optical rotation, as compared to oxyhemocyanin (Table 1).

It is apparent, therefore, that in the oxygenation of hemocyanin, in which an O_2 molecule is bound at a specific localized site, the protein fabric is affected in a very extensive fashion. Such a possibility has also been suggested for hemoglobin, by Haurowitz[12] to account for crystallographic differences between ferro- and oxyhemoglobin, and by Wyman and Allen[13] and by St. George and Pauling[14] as an explanation of the co-operative interactions in the uptake of successive O_2 molecules. From the present experiments it is clear that substantial changes in topographical or structural configurations occur even in the absence of porphyrin prosthetic groups and even when the binding sites do not show any co-operative interactions. The very act of taking up an oxygen molecule makes itself felt over wide regions of the macromolecule.

The nature of these configurational changes is still obscure. The general trend has been to attribute them to steric displacements or rearrangements in polypeptide chains. It is now clear that these steric effects, if they exist, do not depend on the presence of a large porphyrin moiety. It is perhaps pertinent to point out also that the direction and magnitude of the change of optical rotation upon oxygenation are similar to those observed on denaturation of proteins and hence would imply a substantial increase in disorganization of the macromolecule. Nevertheless, oxygenation-deoxygenation processes can be carried through many cycles without appreciable change in the properties of the protein.

We should like to consider, therefore, an alternative model which attributes changes during oxygenation to modifications in the "frozen water"[15-19] of the hydration lattice of the oxygen-carrying protein. From this point of view, the binding site of the deoxygenated protein may be visualized as having an aqueous covering sheath whose lattice structure is determined by the nature of the residues (and metal) at the active site and by the amino acid side chains in the neighborhood of the site. Under appropriate conditions—for example, hemocyanin above its isoelectric point, or hemoglobin at any pH—these crystalline water islands may be sufficiently extensive that they merge into an interwoven lattice. The attachment of an oxygen molecule to the binding site would produce an incongruity in this lattice, because of the larger size[14] of O_2 as compared to H_2O and because of the differences in hydrogen-bonding properties. As a result, the hydration "iceberg" might be disturbed for an appreciable distance around the active site. This disturbance could affect the hydration structure of neighboring sites and hence change their affinity toward oxygen molecules. Simultaneously, it would manifest itself by changes in optical rotation. Changes in degree of interaction between sites of hemocyanin due to changes in pH may be attributed to changes in hydration as the charge of the protein is varied.

In terms of changes in the structure of the hydration lattice, one could also understand why so many different substances affect the affinity, and hence the shape and position of oxygenation curves, of the oxygen-carrying pigments. For hemoglobin these substances may vary from presumably innocuous salts[20] which affect only the ionic strength, to specific reagents such as mercurials[21] which combine with mercaptan groups, to generally reactive substances such as formaldehyde.[22] All these substances can *increase* the oxygen affinity of hemoglobin, and they affect the degree of interaction between sites; all these substances would also modify the lattice structure of the aqueous hydration sheath.

From this point of view the observed dielectric behavior of hemoglobin also seems reasonable. It has been reported by Takashima[23] that the high-frequency dielectric increment of hemoglobin changes with degree of oxygenation. As has been emphasized by Jacobson,[24] dielectric behavior reflects the character of the hydration sheath of a macromolecule. It is only reasonable, then, that binding of oxygen should change the dielectric properties if it affects the water lattice of the protein molecule.

It would seem, therefore, that the character of the "frozen" water on the surface of the protein may play an important role in determining the behavior of the oxygen-carrying pigments.

This investigation was supported in part by a research grant (RG-4134) from the National Institutes of Health, United States Public Health Service.

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