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Functional Properties of Chemically Modified Hemocyanin. Fixation of Hemocyanin in the Low and the High Oxygen Affinity State by Reaction with a Bifunctional Imido Ester†

Roel van Driel* and Ernst F. J. van Bruggen

ABSTRACT: Hemocyanin of *Helix pomatia* is a respiratory protein with a molecular weight of 9×10^6; it contains 180 oxygen binding sites. The reaction of hemocyanin with the bifunctional reagent dimethyl suberimido ester, which reacts with amino groups,has been studied. Up to 75% of the amino groups can be modified without inactivation of oxygen binding sites or dissociation of the protein. It appears that hemocyanin can be fixed in a state with low oxygen affinity by modification of the deoxy protein, and in a state with high oxygen affinity by modification of the oxy protein. Using conditions under which native hemocyanin binds oxygen cooperatively (Hill coefficient 2.9), modification of deoxy- and oxyhemocyanin yields derivatives with different oxygen affinities (P_{50} = 10 and 2.2 mm, respectively). Both the deoxy and oxy derivatives show strongly reduced cooperativity (Hill coefficients 1.4 and 1.1, respectively). Modification of oxy- and deoxyhemocyanin subunits (molecular weight one-tenth of the native protein), which bind oxygen noncooperatively, results in derivatives with oxygen binding properties identical with those of unmodified subunits. Parallel experiments have been carried out with a unifunctional reagent, methyl acetoimido ester. Modification of partially oxygenated hemocyanin under conditions at which the protein binds oxygen cooperatively yields derivatives with reduced cooperativity (Hill coefficients 1.1–1.2) and an oxygen affinity depending on the oxygen saturation at which modification had been carried out. The results are consistent with a simple two-state model for the cooperativity of oxygen binding by these giant hemocyanin molecules.

Hemocyanin is a very large respiratory protein which occurs freely dissolved in the hemolymph of the Roman snail, *Helix pomatia*. Its molecular weight is 9×10^6, and it contains about 180 oxygen binding sites. The protein can be dissociated into ten equal subunits (Konings *et al.*, 1969b), each containing a small number of very large polypeptide chains (Brouwer and Kuiper, 1973). Under the right conditions, oxygen binding is cooperative. Evidence has been presented that cooperativity may be interpreted as an oxygen-linked change from a state with low oxygen affinity, predominant at low oxygen saturation, to a state with high oxygen affinity at high oxygen saturation (Er-el *et al.*, 1972; Van Driel, 1973; Van Driel *et al.*, 1974).

The present paper reports the use of a bifunctional, cross-linking agent, dimethyl suberimido ester,[1] introduced by Davies and Stark (1970). This reacts with amino groups, forming amidine groups (Wold, 1967). Extensively modified hemocyanin retains its ability to combine reversibly with oxygen.

The aim of this study was to investigate oxygen linked conformational changes by cross-linking the protein at various degrees of oxygen saturation, and subsequent analysis of the oxygen binding properties of the hemocyanin derivatives obtained.

Materials and Methods

Helix pomatia α-hemocyanin was isolated, stored, and regenerated as described previously (Konings *et al.*, 1969a; Van Driel, 1973).

Protein concentration was determined routinely by measuring the absorbance at 278 nm in 0.1 M borate buffer (pH 9.3) (Heirwegh *et al.*, 1961). Under these conditions, unmodified hemocyanin dissociates into subunits. However,

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[1] Abbreviations used are: DSI, dimethyl suberimido ester hydrochloride; MAI, methyl acetoimido ester hydrochloride; DSI-deoxy-Hc, the product of reaction of DSI with deoxyhemocyanin (regardless of the oxygenation state of the product); likewise, DSI-oxy-Hc, MAI-deoxy-Hc, and MAI-oxy-Hc.

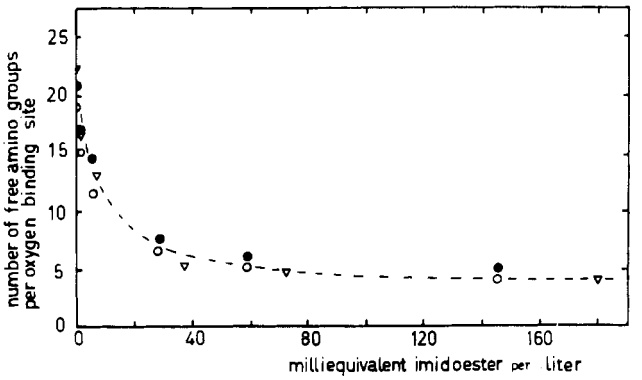

FIGURE 1: Number of free amino groups per oxygen binding site as a function of imido ester concentration. Reaction conditions are described in the text. (●) Dimethyl suberimido ester, in the presence of 10 mM $CaCl_2$; (▽) methyl acetoimido ester, in the presence of 10 mM $CaCl_2$; (○) dimethyl suberimido ester, in the absence of $CaCl_2$.

cross-linked undissociated hemocyanin fails to dissociate, and consequently light scattering contributes significantly to the optical density. Thus, the optical density at 278 nm of cross-linked undissociated hemocyanin was found to be 15% higher than that of unmodified hemocyanin (protein concentrations determined by amino acid analysis). Oxygen binding curves were determined at 20° as described before (Konings *et al.*, 1969a). All buffers contained 0.10 M triethanolamine; NaCl was added up to a ionic strength of 0.10 (Bates, 1954).

DSI was prepared as described by Davies and Stark (1970), mp 219–221°; MAI was prepared according to Hunter and Ludwig (1962), mp 96–99°. The reaction of hemocyanin with DSI or MAI was carried out at pH 8.5 in the absence or presence of $CaCl_2$ (10 mM) at a protein concentration of 5 mg/ml. The reaction was initiated by adding a small volume of a freshly prepared imido ester solution (about 50 mg/ml) in 1 M triethanolamine buffer (pH 8.5) to a final concentration of about 40 mequiv of imido ester groups/l., which is a 20-fold excess over protein amino groups. Modification of oxygen free or partially oxygenated protein was carried out in a tonometer; oxygen-free imido ester solution was injected through a rubber cap. After incubation for 4 hr at 20°, the protein solution was dialyzed against buffer of pH 8.5. From the absorbance at 346 nm, read after addition of reagent, the fractional oxygen saturation at which cross-linking was being carried out was calculated.

The extent of modification of protein amino groups was determined by reaction of the residual free amino groups with trinitrobenzenesulfonic acid (Merck, analytical grade) as described by Habeeb (1966).

Polyacrylamide gel electrophoresis in the presence of sodium dodecyl sulfate was carried out as described by Brouwer and Kuiper (1973); 4% gels with 2.5% cross-linking were used.

Results

Helix pomatia α-hemocyanin can be extensively modified by the reaction with DSI and MAI. Up to about 16 out of 20 amino groups per oxygen binding site reacted with imido ester (Figure 1). The univalent MAI and the bifunctional DSI reacted with hemocyanin to about the same extent. No significant difference in reactivity of liganded and ligand-free hemocyanin has been observed. The following functional studies were carried out with hemocyanin that had been modified with DSI or MAI at a concentration of about 20 and 40 mM, respectively, resulting in blocking of about 75% of the amino groups.

Reaction of Hemocyanin with DSI in the Presence of $CaCl_2$. In triethanolamine buffer (pH 8.5), ionic strength 0.1, in the presence of 10 mM $CaCl_2$, only undissociated hemocyanin is present. Modification with the bifunctional reagent DSI did not change the sedimentation coefficient, which is about 100 S. If $CaCl_2$ is removed by dialysis, native hemocyanin dissociates into ten equal subunits with a sedimentation coefficient of about 20 S (Konings *et al.*, 1969b), whereas the sedimentation coefficient of DSI treated hemocyanin does not change (Figure 2). When subjected to electrophoresis on polyacrylamide gels in the presence of sodium dodecyl sulfate, no DSI treated hemocyanin penetrated the gel. Under identical conditions, untreated hemocyanin migrated considerably (Brouwer and Kuiper, 1973). Apparently, cross-links between the polypeptide chains are formed during the reaction with DSI. No difference between undissociated native hemocyanin (in the presence of $CaCl_2$) and cross-linked hemocyanin has been observed in the electron microscope.

In the presence of $CaCl_2$ the native protein binds oxygen cooperatively; the Hill coefficient (n_H) is 2.9, the oxygen affinity, expressed as the oxygen pressure at 50% saturation

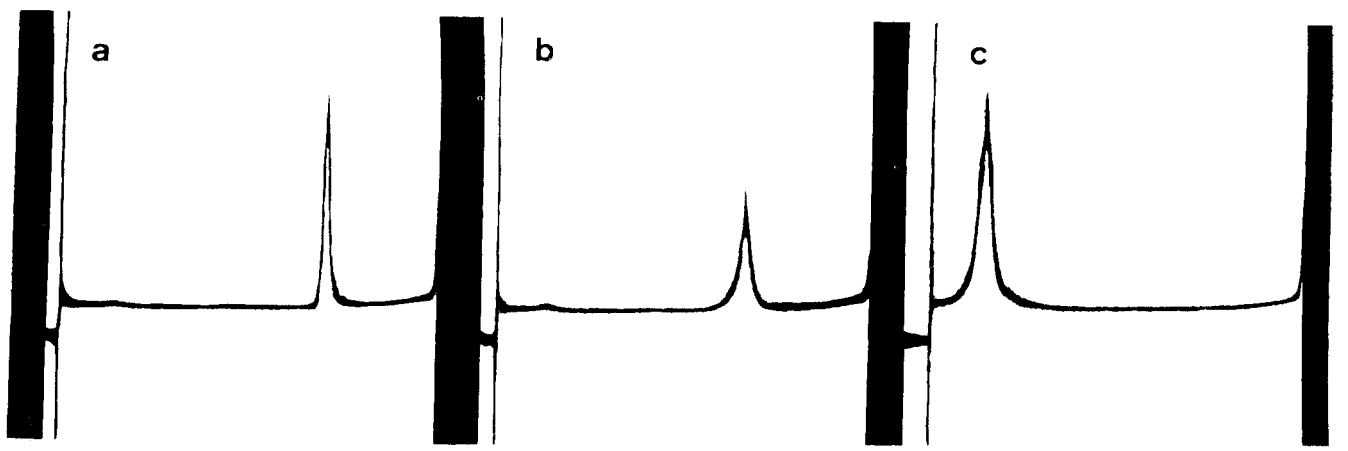

FIGURE 2: Sedimentation velocity analysis in triethanolamine buffer, ionic strength 0.1, pH 8.5, 20°. (a) DSI-oxy-Hc, in the presence of 10 mM $CaCl_2$; (b) DSI-oxy-Hc, in the absence of $CaCl_2$; (c) unmodified hemocyanin, in the absence of $CaCl_2$. Schlieren patterns were obtained after 15, 16.5, and 22 min at 37,020 rpm, respectively.

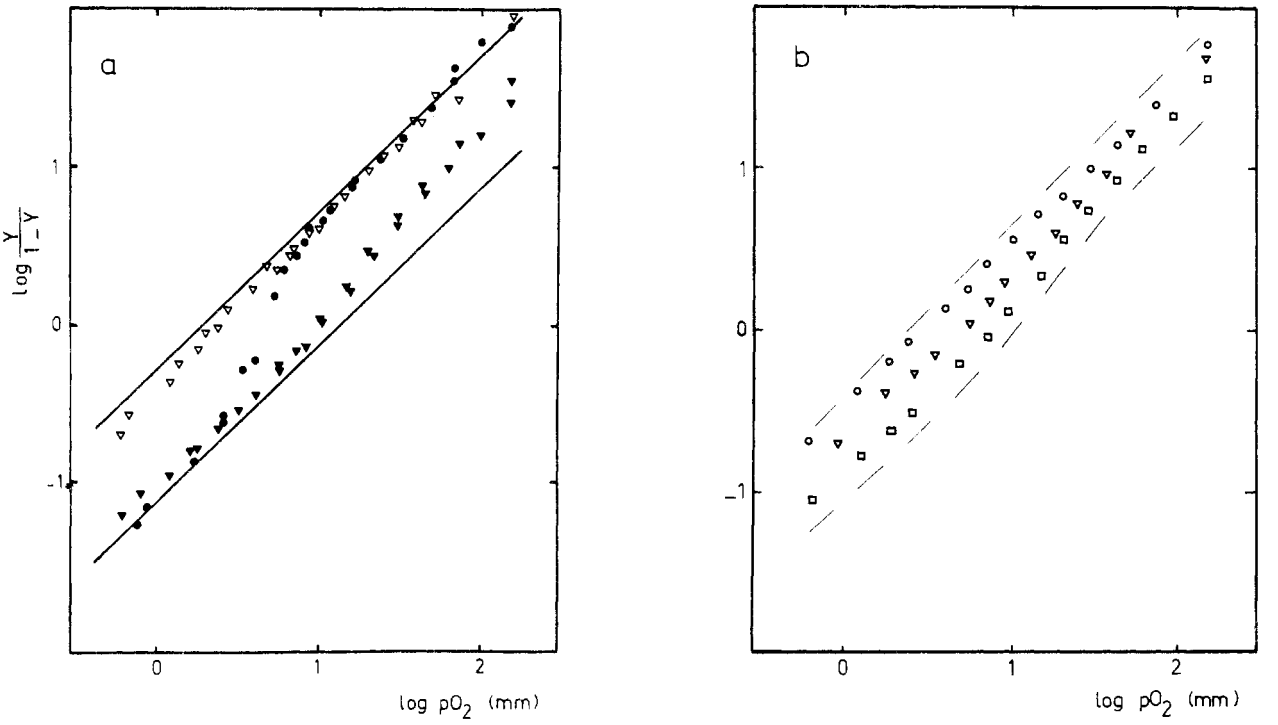

FIGURE 3: Hill plot of oxygen binding curves of hemocyanin, cross-linked in the presence of $CaCl_2$. Conditions: triethanolamine buffer (pH 8.5), ionic strength 0.1, 10 mM $CaCl_2$, 20°. (a) (▼) DSI-deoxy-Hc; (▽) DSI-oxy-Hc; (●) unmodified hemocyanin. The lines represent the hypothetical binding curves of the low and high affinity states of the protein. (b) Hemocyanin cross-linked at various fractional saturations: (□) $Y = 0.26$; (▽) $Y = 0.55$; (○) $Y = 0.73$. The broken lines indicate the binding curves of DSI-deoxy-Hc and DSI-oxy-Hc for comparison with Figure 3a. Y, fractional saturation; pO_2, oxygen pressure.

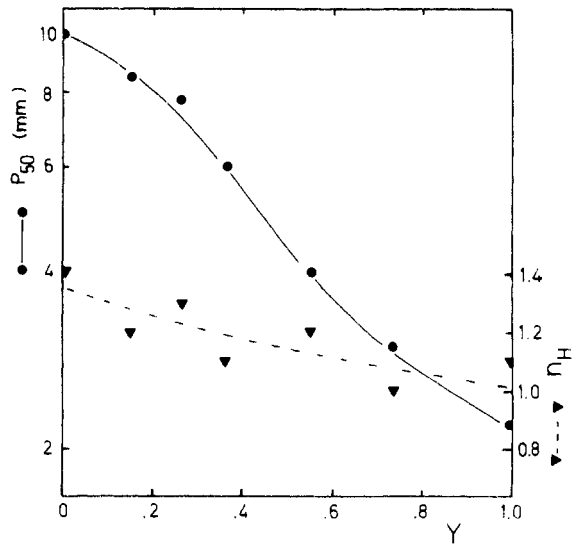

FIGURE 4: Oxygen pressure at half-saturation (P_{50}) and Hill coefficient (N_H) of hemocyanin cross-linked at various fractional saturations with oxygen (Y), in the presence of $CaCl_2$. Conditions as in Figure 3.

Table I: Oxygen Binding Properties of Modified Hemocyanin; Triethanolamine Buffer (pH 8.5), 20°.

Reagent	Hemocyanin	$CaCl_2$ (10 mM) Present during Modification	Oxygen Binding Parameters: In the Presence of 10 mM $CaCl_2$ P_{50} (mm)	n_H	In the Absence of $CaCl_2$ P_{50} (mm)	n_H
DSI	Deoxy-Hc	Yes	10	1.4	13	1.0
DSI	Oxy-Hc	Yes	2.2	1.1	6.5	1.0
MAI	Deoxy-Hc	Yes	3.7	1.5		
MAI	Oxy-Hc	Yes	2.4	1.3		
DSI	Deoxy-Hc	No			4.5	0.82
DSI	Oxy-Hc	No			4.3	0.86
None			6.0	2.9	4.0	0.83

(P_{50}) is 6 mm. Cross-linking was carried out at various degrees of oxygen saturation. Oxygen binding curves of the resulting hemocyanin derivatives are shown in Figure 3, n_H and P_{50} values are summarized in Table I and Figure 4. Apparently, cross-linking reduces the cooperativity of oxygen binding, but did not completely abolish it. The oxygen affinity of cross-linked hemocyanin increased continuously with the oxygen saturation at which cross-linking was carried out. Essentially the same results were found if hemocyanin was cross-linked at a twofold lower DSI and/or protein concentration. The oxygen binding capacity, expressed as the difference between the extinction coefficients at 346 nm of the completely liganded and ligand-free protein, was not affected by the modification.

If $CaCl_2$ is removed, the native protein dissociates completely into ten equal subunits; oxygen binding becomes noncooperative (n_H = 0.8–0.9) and the oxygen affinity increases to a P_{50} of 4 mm. On the other hand, removal of $CaCl_2$ decreased the oxygen affinity of DSI-oxy-Hc and DSI-deoxy-Hc to a P_{50} of 6.5 and 13 mm, respectively (Figure 5 and Table I). The Hill coefficient at 50% saturation was close to 1.0, but the slight upward curvature of the Hill plot at high oxygen concentrations, which was found for

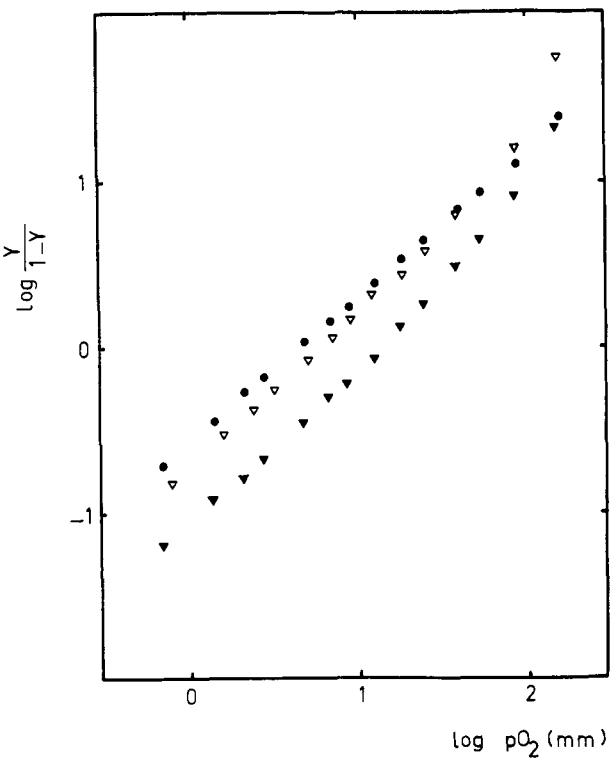

FIGURE 5: Hill plot of oxygen binding curves of hemocyanin, cross-linked in the presence of $CaCl_2$. Conditions: triethanolamine buffer (pH 8.5), ionic strength 0.1, no $CaCl_2$, 20°. (▼) DSI-deoxy-Hc; (▽) DSI-oxy-Hc; (●) unmodified hemocyanin. *Y*, fractional saturation with oxygen; pO_2, oxygen pressure.

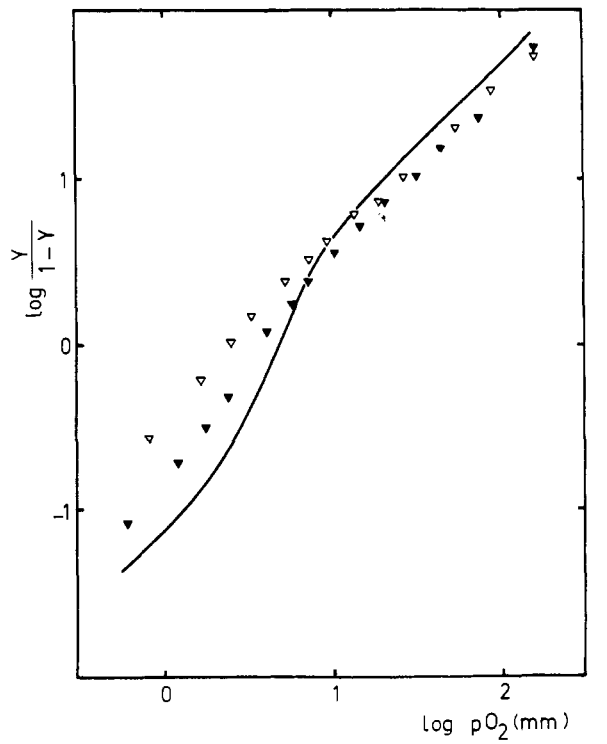

FIGURE 6: Hill plot of oxygen binding curves of hemocyanin, modified with MAI in the presence of $CaCl_2$. Conditions as in Figure 3. (▼) MAI-deoxy-Hc; (▽) MAI-oxy-Hc; the line represents the binding curve of unmodified hemocyanin (from Figure 3a). *Y*, fractional saturation with oxygen; pO_2, oxygen pressure.

both derivatives, is significant (Figure 5).

Modification of Hemocyanin with MAI in the Presence of $CaCl_2$. After modification of hemocyanin with the unifunctional reagent MAI in the presence of $CaCl_2$ to about the same extent as with DSI, only undissociated protein was present, but after removal of $CaCl_2$ by dialysis, MAI treated hemocyanin had dissociated into subunits. Oxygen binding by MAI-deoxy-Hc and MAI-oxy-Hc showed decreased cooperativity and increased oxygen affinity as compared to the unmodified protein (Figure 6, Table I).

When MAI-deoxy-Hc and MAI-oxy-Hc were incubated with DSI, the number of free amino groups did not decrease significantly and the oxygen binding properties did not change. This indicates that both imido esters react with the same amino groups of the protein.

Modification of Hemocyanin with DSI in the Absence of $CaCl_2$. In triethanolamine buffer (pH 8.5), ionic strength 0.1, in the absence of $CaCl_2$, hemocyanin is dissociated and oxygen binding is noncooperative. Reaction of the subunits with DSI resulted in about the same degree of modification as found for the undissociated protein (Figure 1). The oxygen binding curves of subunits cross-linked under both liganded and ligand free conditions were nearly identical with the binding curve found for unmodified subunits (Table I).

Discussion

Imido esters react with amino groups with a high degree of specificity under mild conditions. Extensive modification results in no change in the net charge of the protein since the reaction product, the amidine, retains the positive charge of the original amino group under the conditions of the modification (Wold, 1967). The unifunctional MAI and the bifunctional DSI react with hemocyanin to about the same extent (Figure 1), both under liganded and ligand free conditions. With the trinitrobenzenesulfonic acid method (Habeeb, 1966) we found that *Helix pomatia* hemocyanin contains about 20 amino groups per oxygen binding site (molecular weight 50,000). This is in reasonable agreement with Dijk *et al.* (1970), who found 17.2 lysine groups per 50,000 molecular weight by amino acid analysis. It is remarkable that up to about 75% of the amino groups may be modified without dissociation of the protein or destruction of oxygen binding sites.

DSI treated hemocyanin failed to dissociate into subunits upon removal of calcium ions or into its polypeptide chains by sodium dodecyl sulfate, and sedimentation analysis showed no evidence for cross-linking between molecules. Reaction with MAI, on the other hand, did not interfere with the dissociation of hemocyanin. We conclude that DSI cross-links hemocyanin intramolecularly, both between subunits and between the polypeptide chains within a subunit. Cross-linking of liganded and ligand free hemocyanin under conditions at which native hemocyanin binds oxygen cooperatively (pH 8.2, in the presence of $CaCl_2$) results in derivatives with different ligand binding properties. The Hill plots of the oxygen binding curves of DSI-deoxy-Hc and DSI-oxy-Hc approach the asymptotes of the Hill plot for the unmodified protein at low and high oxygen concentrations, respectively, as shown in Figure 3a. The asymptotes have been interpreted as the hypothetical binding curves of the protein in a state with low and high ligand affinity, respectively, the cooperativity of oxygen binding being the result of an oxygen linked transition from the low to the high affinity state (Er-el *et al.*, 1972, Van Driel, 1973). From a mechanistic point of view, these data may mean that cross-linking largely prevents the conformational changes which are responsible for the homotropic interactions between the oxygen binding sites. In terms of the well-known model of

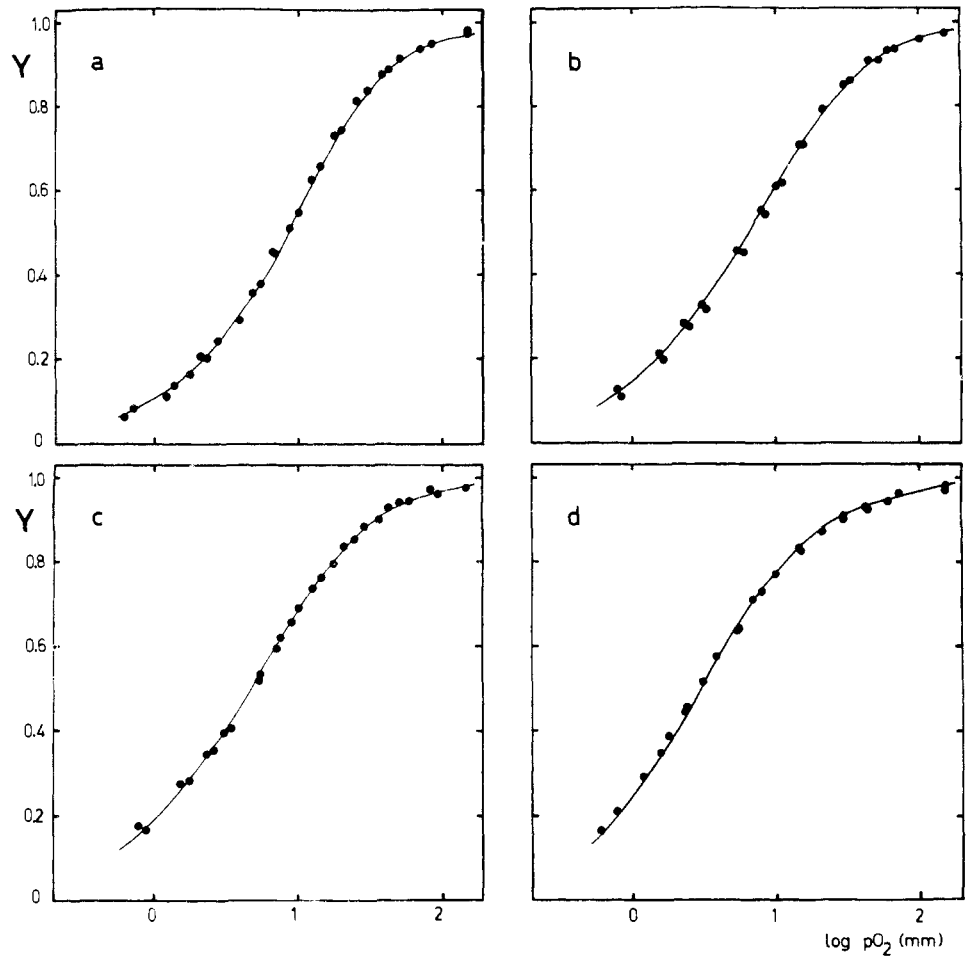

FIGURE 7: Comparison between calculated (from the ratios given in Table II) and experimental oxygen binding curves of hemocyanin cross-linked in the presence of $CaCl_2$ at various fractional saturations with oxygen: (a) $Y = 0.15$; (b) $Y = 0.26$; (c) $Y = 0.55$; (d) $Y = 0.73$. Experimental conditions as in Figure 3. Y, fractional saturation with oxygen; pO_2, oxygen pressure.

Monod *et al.* (1965), cross-linking drastically changes the allosteric constant L_0, the equilibrium constant between the low and high affinity state in the absence of oxygen.

In the absence of $CaCl_2$, oxygen binding is noncooperative; no oxygen-linked structural transition takes place. The Hill coefficient is about 0.85, suggesting some heterogeneity in the oxygen binding sites (Figure 5). As expected, under these conditions cross-linking of deoxy- and oxyhemocyanin yields derivatives with binding properties very similar to those of the unmodified protein (Table I).

Modification of the oxy protein with the unifunctional reagent MAI yields a derivative whose properties are very similar to those of DSI-oxy-Hc (Table I). The properties of MAI-deoxy-Hc are intermediate between those of unmodified and MAI-oxy-Hc (Figure 5). The binding curves coincide at high oxygen saturation. Thus, it seems that modification with the unifunctional reagent shifts the equilibrium between the hypothetical low and high affinity states toward the latter. Comparison with the properties of DSI-deoxy-Hc strongly suggests that cross-linking—and not the mere modification of protein amino groups—is responsible for fixation of DSI-deoxy-Hc in the low affinity state.

In previous papers evidence has been presented that the ligand binding characteristics of the dissociated protein (in the absence of $CaCl_2$) and the high affinity state of the cooperative protein (in the presence of $CaCl_2$) are similar. These structures are therefore assumed to be similar, whereas the properties of hemocyanin in the low affinity state are quite different (Er-el *et al.*, 1972; Van Driel, 1973). This is supported by the observation that, if $CaCl_2$ is removed after cross-linking in the presence of $CaCl_2$, DSI-oxy-Hc and the (dissociated) unmodified protein have similar oxygen binding properties, whereas DSI-deoxy-Hc has a much lower oxygen affinity (Figure 5).

As mentioned earlier, the cooperativity of oxygen binding may be interpreted as an oxygen-linked transition from a state with low oxygen affinity to one with high affinity. An

Table II: Interpretation of Oxygen Binding Properties of Cross-Linked, Partially Oxygenated Hemocyanin in Terms of a Mixture of DSI-deoxy-Hc and DSI-oxy-Hc.

Cross-linking at Fractional Saturation	Composition of the Hypothetical Mixture[a]	
	DSI-deoxy-Hc %	DSI-oxy-Hc %
0	100	0
0.15	86	14
0.26	78	22
0.36	66	34
0.55	46	54
0.73	16	84
1.0	0	100

[a] Found by trial and error.

important question is whether, under equilibrium conditions, any structures with intermediate oxygen affinity are populated significantly. Recent kinetic investigations of the cooperative oxygen binding process suggest that intermediate states do not play a significant role (Van Driel *et al.*, 1974). Calculations of Wyman (1969) showed that in systems with a high number of binding sites intermediate conformations may be destabilized as compared to both extreme states. We have tried to trap the protein when it binds oxygen cooperatively, in possible intermediate states by cross-linking partially oxygenated hemocyanin. As shown in Figure 4, the oxygen affinity of cross-linked hemocyanin increased with increasing oxygen saturation at which cross-linking was carried out. The Hill coefficient was slightly higher than 1.0.

If the system can be described by a simple two-state model, it should be possible to describe the oxygen binding curves of cross-linked hemocyanin as mixtures of DSI-deoxy-Hc and DSI-oxy-Hc. Using the hypothetical ratios given in Table II, the fit between the calculated and observed binding curves is surprisingly good (Figure 7). This supports the two-state hypothesis for cooperative oxygen binding, although, it might be that intermediate states are not cross-linked, for instance because DSI might react more rapidly with both extreme states in equilibrium with the intermediates. The possibility to fix hemocyanin in states with different affinities may facilitate investigations of the structural changes that underlie the process of cooperative ligand binding by these giant proteins.

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The Solution Conformation of Nicotinamide Mononucleotide: A Quantitative Application of the Nuclear Overhauser Effect†

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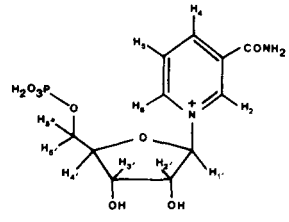
ABSTRACT: Torsion about the glycosidic linkage in nicotinamide mononucleotide has been investigated by quantitative application of the nuclear Overhauser effect. These measurements show that the syn ($\chi \simeq 20°$) and anti ($\chi \simeq 200°$) conformers of the title compound are isoenergetic, or nearly so, and interconverting rapidly. The syn/anti partition is not measurably affected by either changes in pH or temperature.

Extensive studies of the pyridine nucleotides, directed toward the elucidation of their solution conformation, have been carried out (for a leading reference, see Blumenstein and Raftery, 1973). The intended goal of these studies has been to relate molecular geometry to biological function. Of the various pyridine nucleotides, nicotinamide mononucleotide (NMN^+) has received considerable attention (see Sarma and Mynott, 1973b, and references cited therein), its



NMN^+

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