SC 2259

Hybridization of deoxygenated human hemoglobin

Evidence for conformational differences between deoxygenated and oxygenated hemoglobin has been obtained by several investigators[1], and recently differences between deoxygenated (human) hemoglobin and oxygenated (horse) hemoglobin have been observed by X-ray crystalliography[2]. It has been suggested that inter-chain interactions between unlike chains may be important in maintaining the configuration of deoxygenated hemoglobin[3].

Considerable evidence indicates that the reversible dissociation of human hemoglobin into half molecules at pH 4.7 (see ref. 4) or pH 11 (see ref. 5) results from symmetrical cleavage of the tetramer to yield 2 $\alpha\beta$ sub-units[6,7]. VINOGRAD AND HUTCHINSON[6] suggested that, in these pH ranges, further dissociation of hemoglobin into single polypeptide chains occurs. The concentration of single polypeptide chains must be small, since no direct evidence for the existence of quarter molecules has been obtained from electrophoretic or sedimentation data.

Extensive application of the dissociation of hemoglobin at pH 4.7 has been made in hybridization experiments, as described by ITANO AND SINGER[8]. If a mixture of 2 hemoglobins, one with an α-chain abnormality and the other with a β-chain abnormality, is allowed to stand for a few hours at pH 4.7, and subsequently neutralized, 2 new hemoglobin species may be identified. For example when hybridization is carried out on hemoglobins I and S, the results may be formulated thus:

$$\underset{\text{Hb I}}{\alpha_2^{I}\beta_2^{A}} + \underset{\text{Hb S}}{\alpha_2^{A}\beta_2^{S}} \rightarrow \underset{\text{Hb I}}{\alpha_2^{I}\beta_2^{A}} + \underset{\text{doubly abnormal Hb}}{\alpha_2^{I}\beta_2^{S}} + \underset{\text{Hb A}}{\alpha_2^{A}\beta_2^{A}} + \underset{\text{Hb S}}{\alpha_2^{A}\beta_2^{S}}$$

When such a hybridization experiment is carried out on a mixture of hemoglobin C and I, the 2 new hemoglobins ($\alpha_2^{A}\beta_2^{A}$ and $\alpha_2^{I}\beta_2^{C}$) have the same electrophoretic mobility[9], and only one new hemoglobin (designated here as "new") is observed on electrophoresis of the neutralized mixture. Since deoxygenated hemoglobin S exhibits unique solubility properties, hemoglobin C was utilized as the β-chain variant in the present study.

Such hybridization experiments appeared to offer an opportunity to compare the dissociation of the polypeptide chains of oxygenated and deoxygenated hemoglobin.

The hemoglobin C and $I^{Burlington}$ (ref. 10) utilized for these studies had the electrophoretic properties characteristic of these abnormal hemoglobins. Fingerprinting by BAGLIONI's method[11] showed each to have the characteristic peptides described by others for hemoglobin C (ref. 12) and for hemoglobin I (ref. 13). (From determinations of amino acid sequences, hemoglobin C may be designated $\alpha_2^{A}\beta_2^{6\,lys}$ (ref. 12) and hemoglobin I, $\alpha_2^{6\,asp}\beta_2^{A}$ (ref. 13).) Both the hemoglobin C and the hemoglobin I had O_2-dissociation curves similar to that of hemoglobin A.

Fresh samples of hemoglobins C and I were prepared by starch-block electrophoresis in barbital buffer (pH 8.6), dialyzed against water and concentrated as previously described[14]. A mixture of approximately equal amounts of purified hemoglobins C and I of concentration of 2 or 3 g % was divided among 4 tonometers which were rotated at 0° and treated as follows:

(1) Control (O_2). O_2 passed through tonometer for 1 h, then 0.2 vol. of 1 M phosphate buffer (pH 7.5) was added.

(2) Recombined (O_2). O_2 passed through tonometer for 1 h, then 0.1 vol. of 2 M acetate buffer (pH 4.7) was added.

(3) Control (N_2). Prepurified N_2 passed through tonometer for 1 h, then 0.2 vol. of 1 M phosphate buffer (pH 7.5) was added anaerobically.

(4) Recombined (N_2). Prepurified N_2 passed through tonometer for 1 h, then 0.1 vol. of 2 M acetate buffer (pH 4.7) was added anaerobically.

Both the rotation of the tonometer and the gas flow were continued for 4 h at 0°. Then 5 vol. of 1 M phosphate buffer (pH 7.5) were added to each tonometer and the hemoglobin solutions were dialyzed in air for 16 h against 200 vol. of 0.02 M phosphate buffer (pH 6.8). After centrifugation, the hemoglobin solution was analyzed by electrophoresis on starch gels[15], and in several experiments the amount of each component was determined after electrophoresis on starch granules[16].

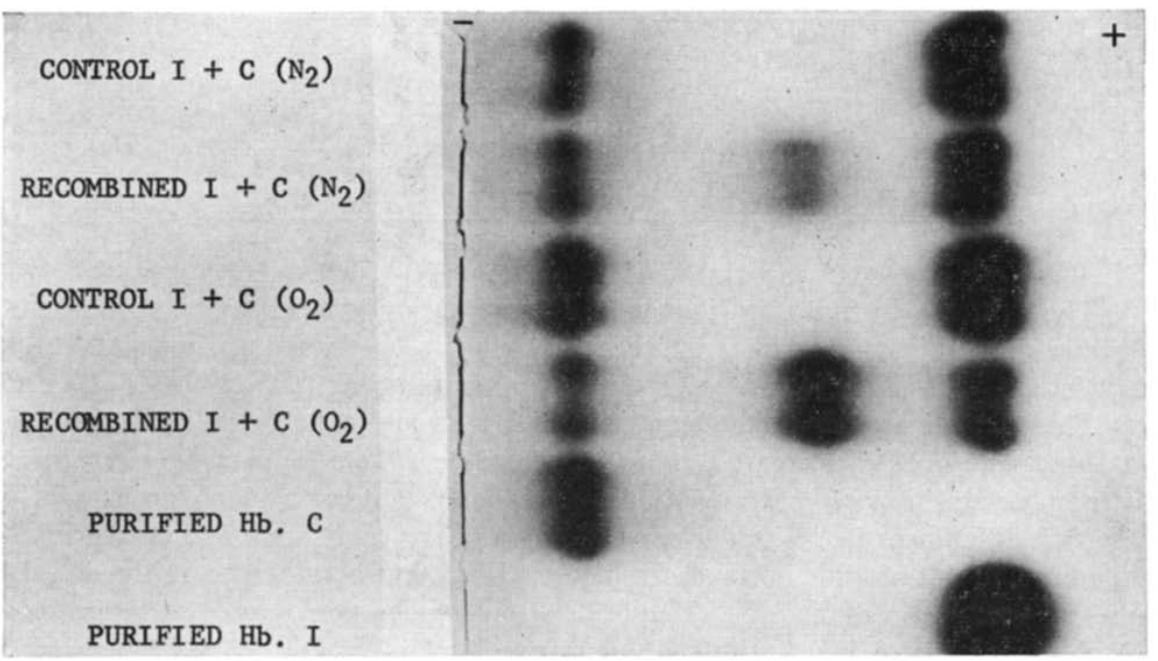

Fig. 1. Vertical starch-gel electrophoresis for 16 h at 4° in borate buffer[15]. Benzidine stain. The decrease in amount of "new" hemoglobin in the deoxygenated hemoglobin mixture compared with the oxygenated is obvious. The amounts of hemoglobins C and I in the control mixture were approximately equal: greater diffusion of hemoglobin I in the gel accounts for the apparent increase in amount of hemoglobin I on vertical starch gels. In this experiment, quantitative determination of the hemoglobin components after elution from starch-block electrophoretic analysis[16] yielded the following results: Control (average of O_2 and N_2): C, 50%; I, 50%. Acidified under N_2: C, 43%; "New", 18%; I, 39%. Acidified under O_2: C, 30%; "New", 43%; I, 27%.

The amount of "new" hemoglobin ($\alpha_2^A \beta_2^A$ and $\alpha_2^I \beta_2^C$) formed when hybridization of hemoglobins C and I was carried out at pH 4.7 was markedly decreased in the deoxygenated sample as compared with the oxygenated sample in all cases. This effect was noted in the starch-gel analyses (Fig. 1) and in the quantitative data obtained from elution from starch blocks (Table I). Considerable variation in the amount of "new" hemoglobin formed was noted in hybridization of both the oxygenated and the deoxygenated mixtures. However, as shown in Table I, decreased hybridization of the deoxygenated hemoglobin mixtures was observed under different experimental conditions, and was particularly evident in the more dilute mixtures of the hemoglobins.

Small amounts of methemoglobin were formed during the experiments: the solutions which were oxygenated and acidified showed the largest amount of methemoglobin but this did not appear quantitatively sufficient to influence the results. No significant differences in hybridization were observed when an oxyhemoglobin C and I mixture was compared with a mixture of the carbonmonoxy derivatives of the same hemoglobins.

TABLE I

HYBRIDIZATION OF OXYGENATED AND DEOXYGENATED MIXTURES OF HUMAN HEMOGLOBINS C AND I

Conditions of hybridization				*"New" hemoglobin (% of total eluted from starch-block electrophoresis[16])*	
Total hemoglobin concn. (g %)	*pH*	*Temp. (°C)*	*Time (h)*	*in O_2*	*in N_2*
2	4.7	0	4	43	19
2	4.7	0	4	17	9
2	5.3	20	2.5	40	16
0.13	4.7	0	4	28	3
0.13	4.7	0	4	19	3

The decrease in hybridization upon deoxygenation was found to be fully reversible since the same amount of hybridization was observed in acidified samples kept for 4 h under N_2 followed by 4 h under O_2 as in those kept for 4 h under O_2 only.

These results provide further evidence for conformational changes in hemoglobin on deoxygenation. The decrease in hybridization in deoxyhemoglobin is best explained by the presence of more or stronger inter-chain bonds in deoxyhemoglobin. Recently observed differences in molecular weight between oxyhemoglobin and deoxyhemoglobin in 2 M salt provide evidence for oxygenation labile bonds between the $\alpha\beta$ sub-units in deoxyhemoglobin[17]. Since hybridization implies not only dissociation into $\alpha\beta$ sub-units but also dissociation into quarter molecules, it follows that the molecular configuration of deoxyhemoglobin also includes oxygenation-labile bonds between α and β chains.

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Zum Mechanismus der mikrosomalen Ascorbinsäure-abhängigen $NADH_2$-Oxidation

Der Frage, ob Dehydroascorbinsäure imstande ist, die mikrosomale $NADH_2$-Oxidation zu beschleunigen, kommt für den Mechanismus der Ascorbinsäurewirkung grundsätzliche Bedeutung zu. Die hierin aufgetretene Diskrepanz zwischen den von uns[1,2] und den unlängst von FRUNDER, BLUME UND KLUGE[3] veröffentlichten Befunden veranlasste uns deshalb, erneut die Identität der eingesetzten Dehydroascorbinsäure zu prüfen und mit der so definierten Substanz die $NADH_2$-Oxidation zu untersuchen. Dazu verwendeten wir als Dehydroascorbinsäure Praeparate aus den Forschungslaboratorien der Firmen E. Merck AG, Darmstadt, und der Deutschen Hoffmann–La Roche AG, Grenzach-Baden. Beide Substanzen zeigten identische Ultraviolett- und Infrarotspektren. Darin ist neben zahlreichen anderen Gruppenschwingungen besonders die scharfe, nach kürzeren Wellen (5.62 μ) verschobene Carbonylabsorption der für die Dehydroascorbinsäure charakteristischen Diketo-γ-Lacton-Gruppierung für die Konstitution beider Praeparate beweisend. Die aus Dehydroascorbinsäure durch Reduktion mit H_2S in neutraler Lösung gewonnenen und durch Umkristallisieren gereinigten Ascorbinsäureproben ergaben die für Ascorbinsäure typischen Ultraviolett- und Infrarotspektren.

Zur Bestimmung der enzymatischen Ascorbinsäure-abhängigen $NADH_2$-Oxidation wurde die Extinktionsabnahme über die Zeit registriert. Wie Tabelle I zeigt, sind die spezifischen Aktivitäten für authentische Ascorbinsäure und für Ascorbinsäure, gewonnen durch Reduktion aus Dehydroascorbinsäure, annähernd gleich. Demgegenüber lässt sich mit Dehydroascorbinsäure keine, bzw. lediglich in den angereicherten Proteinfraktionen eine geringe Aktivierung der $NADH_2$-Oxidation erreichen. Prinzipiell gleiche Befunde wurden mit dem von FRUNDER, BLUME UND