RAPID STRUCTURAL CHANGES IN HUMAN HEMOGLOBIN STUDIED BY LASER PHOTOLYSIS.

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Summary : The removal of carbon monoxide and oxygen from liganded hemoglobin by a short laser pulse yields a transient species which is converted into the stable deoxy form within about 100 nsec. It is postulated that the observed spectral change corresponds to modifications of tertiary structure associated with changes in electronic spin-phonon coupling upon ligand removal.

Introduction

Hemoglobin free from ligand may be obtained by photodissociation of carbonmonoxy- or oxy-hemoglobin, although only with a low quantum efficiency from the latter compound. The original liganded form is restored by reaction of the free hemoglobin with the free ligand. In an early flash-photolytic study, Gibson (1) attributed a rapid phase observed in this recombination to an unstable species which was originally thought to be free hemoglobi retaining the quaternary structure of the liganded form for a short period of time after rapid removal of ligand. The characterization of the "rapidly reacting form" has since been the subject of many stimulating experiments, all of which are based on the measurement of the recombination reaction (2, 3, 9).

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In the present work we have studied the free hemoglobin produced by photolysis during a short interval of time before any noticeable recombination with ligand took place. The photolysis was produced by a laser flash at 529 nm of total duration 50 nsec and the product was observed during a further 300 nsec. Measurements were performed on carboxy and oxy derivatives of human hemoglobin, those of the valency hybrids (4), isolated chains (α and β), on free carboxyheme and also on free hemoglobin and methemoglobin.

Materials and methods

Hemoglobin and its various derivatives were prepared by standard procedures. Free carboxyheme was prepared by dithionite reduction of an alkaline solution (o.1 M NaOH) of ferriheme under saturating carbon monoxide. Except for carboxyheme, all measurements were performed in 0.1 M potassium phosphate buffer (pH 6.9). Hemoprotein concentrations were between 7 and 8.6×10^{-5} M (on heme basis).

Excitation light was obtained by second harmonic generation of the output from a Q-switched Nd glass laser (Compagnie Générale d'Electricité, type VD 230). The energy of the second harmonic was 0.1 - 1 Joule in the present study. The solution was contained in a silica cell for spectrophotometry with polished sides of section 1x10 mm or 5x10 mm. A device for degassing of the solution and saturation by CO or O_2 at 1 atm was sealed directly to the cell. The laser light was projected at normal incidence on one of the short sides of the cell. Transient light absorption changes in the solution were measured photoelectrically in a crossed-beam arrangement over a 2 mm wide section of the irradiated part of the solution, using a Xenon flash lamp as monitoring light source. The intensity of the flash was constant over 20 µsec after the laser pulse. The monitoring light was rendered monochromatic (bandwidth 2.6 nm) and was detected by a photomultiplier tube connected to an oscilloscope with photographic recording. The time resolution was ~10 nsec.

Results and discussion

Fig. 1 shows oscilloscope traces representing transient transmittance changes during the first 300 nsec after the laser pulse in some typical photolysis experiments. The laser flash has no measurable effect on the transmittance of a solution of ligand free hemoglobin or on that of a solution of

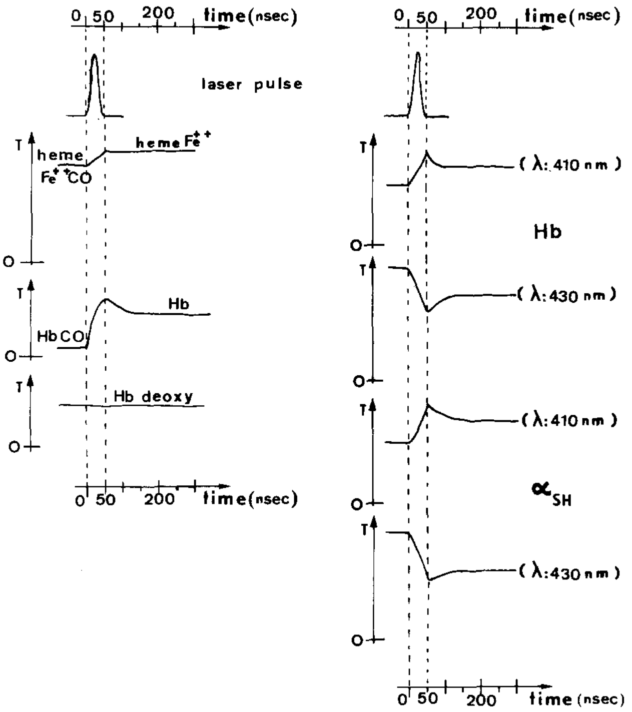

Figure 1. Oscilloscope traces of representative photolysis experiments showing laser-induced transmittance changes, for a few of the compounds studied (see text). Left : CO-derivatives; Transmittance (T) at 420 nm. Right : O_2 derivatives; T at 430 nm and 410 nm.

methemoglobin, as may be expected since the light energy does not produce any chemical change in these cases. On the other hand, for all samples undergoing photolysis, the laser flash is followed by an increase or a decrease in transmittance, depending on the observation wavelength. For all oxygen or carbon monoxide bound hemoproteins studied, the oscilloscope trace reveals a transmittance change during the laser pulse, then a return to a constant intermediate level over a period of 50 - 100 nsec after the complete extinction of the flash. For carboxyheme, on the contrary, a constant level is attained immediatly after the flash. The absorption spectrum at the end of the observation period was calculated for each experiment from the stationary transmittance before the flash. This spectrum (Fig. 2) corresponds to that of totally ligand-free ferroheme or hemoprotein (Hb, α , β , valency hybrids) when the starting material was the carbonmonoxy derivative; photolysis of HbO_2 yielded, at the stationary level,as much as 30% Hb in spi of the very low quantum yield of photodissociation (0.008 as

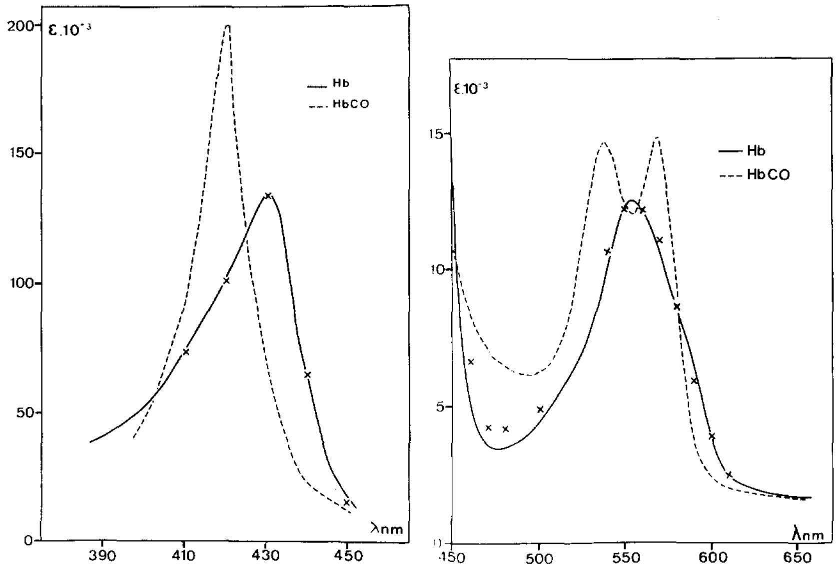

Figure 2. Absorbance values (**X**) of the product at 200 nsec after photolysis of HbCO as calculated from the stationary transmittance level at different monitoring wavelengths. Continuous and broken lines are the absorption spectra of Hb and HbCO, respectively.

compared to 0.27 for HbCO).

Several broad conclusions can be derived from the results depicted in Fig. 1 and 2. Firstly, it is clear, even at the end of the observation period (300 nsec), that the photolysed protein was ligand-free, no perceptible fraction had yet combined with free ligand (CO or O_2). This conclusion is based primilarly on the absorption spectrum of the product[†], but is supported by a reference to the published values of the recombination velocity constants (5) including those for the "quickly reacting form". The recombination reaction could be observed over a period of 20 µsec; at the end of this period still only 5% HbCO had reappeared. Secondly, in the case of all proteins, the oscilloscope trace revealed a change in the transient absorption spectrum occuring within about 100 nsec after the photolysis. In view of the significance that this phenomenon

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[†] The readings are thought to be accurate to within 10%. This precision is insufficient to detect small departures from the stable deoxy spectrum as might be caused by the presence of "reactive dimers" (6), or other reactive species believed to absorbe 15% less in the Soret region.

Admitting that the deoxy dimer produced by photolysis is spectrally equivalent to the quickly reactive species, its presence would cause only a 2% fall of absorbance at 430 nm.

may assume, we have carefully considered various arguments in order to ascertain whether this optical density change represents a real feature of the reaction or might arise from experimental errors.

The possibility of laser artefacts was very remote, since the effect was never observed with deoxy or methemoglobin; moreover when a spectral change occured (c.f. with HbCO), its direction was found to depend on the observation wavelength. We have also considered the effect of molecular motions of the protein, the relaxation times of which may be of the order of 200 nsec. Indeed, since the laser light is polarized, and the analysis light partially polarized due to the monochromator a rapid transmittance change due to orientation relaxation of the excited protein could possibly occur. Further measurements in which the observation beam was polarized, parallely and perpendicularly to the direction of the laser beam showed that this effect could be neglected.

We therefore conclude that the photolysis of carbonmonoxy or oxy derivative of the hemoproteins studied in this work gives rise to a primary transient species which is transformed into the respective stable deoxy compound within about 100 nsec after formation. It was not possible in this work to obtain a precise description of the absorption spectrum of the transient species because of its short lifetime, of the same order of magnitude as that of the laser pulse. However, the results indicate that the spectrum is slightly but significantly different from that of stable deoxy Hb.

It is tempting to speculate on the nature of the transient species and on its position in the sequence of events that must follow the departure of bound ligand from hemoglobin.

As is well known, carbonmonoxy and deoxy hemoglobin are characterized by structural differences at various levels, quaternary and tertiary. Perhaps the most important is the differences in spin-state of the iron going over from $S = 0$ in carboxy to $S = 2$ in the deoxy form, since it is this difference that must act as a trigger for the overall conformation change.

Early predictions (7) on the effect of this spin change on protein structure have been recently born out by X-ray cristallographic results (8) on two extreme stable forms (oxy and deoxy). It is interesting to concentrate on the dynamics of this transformation and to visualise the events following the rapid removal of ligand from the protein. The liganded form (S=O) is characterized by a given geometry describing the position of the iron atom relative to the porphyrin plane as well as to the rest of the polypeptide chain. The departure of ligand profoundly modifies the electronic spin-lattice vibration (phonon) coupling between the iron atom and the rest of the molecule and must be followed by an overall structural change going over to a new geometry consistent with the new equilibrium in the spin-phonon coupling between unliganded iron and the protein. This process may require a large number of adjustments in the globin and the overall structural change should, as a consequence, be relatively slow. We suggest that the initial spectral transmittance change reflected in the oscilloscope traces immediatly after photolysis of carbonmonoxy or oxy hemoproteins corresponds to such a modification of tertiary structure.

It should be recalled that the spectral change discussed above was obtained for all hemoproteins undergoing photolysis, irrespective of their aggregation states (tetrameric or monomeric, HbCO, αCO, βCO etc...). It is thus unneccessary to consider any part played by the change of quaternary conformation which inevitably follows the tertiary restructuring. Besides, a quaternary conformational change taking place without affecting the spin-phonon coupling exchange energy of the individual chains should not be expected to give rise to a spectral change and may, as a result, go unnoticed. A more detailed study of the rapidly disappearing transient species using a shorter-lasting laser pulse is planned.

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