THE KINETICS OF CARBON MONOXIDE BINDING TO PARTIALLY REDUCED METHEMOGLOBIN

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SUMMARY: The pulse radiolysis technique has been used to study the kinetics of the CO binding to partially reduced methemoglobin. Experiments with horse heart metmyoglobin show that this technique gives results which are in good agreement with those obtained by other methods. The kinetics of the CO binding to partially reduced methemoglobin show two phases, whose amplitudes appear to depend on the degree of reduction in such a way that they can be attributed to methemoglobin molecules with one or two reduced heme groups. In the presence of inositol hexaphosphate the rate of CO binding to partially reduced methemoglobin decreases strongly. With inositol hexaphosphate a slight biphasic behavior is observed independent of the degree of reduction.

Until now the kinetics of the binding of CO to hemoglobin have been studied by rapidly mixing deoxyhemoglobin with CO or by following the CO recombination after removal of the ligand by flash photolysis (1). A new approach to the CO binding kinetics of hemoglobin is offered by the pulse radiolysis technique. Using this technique a methemoglobin solution is irradiated with a short pulse of high energy electrons. The irradiation mainly results in the formation of hydrated electrons, OH and H radicals. Of these primary radicals OH and H can be removed by an appropriate scavenger. The hydrated electrons reduce methemoglobin within a few microseconds. Two secondary processes which are complete in about 500 μs are observed after reduction (2). When the reduction is carried out in the presence of CO, the kinetics of the CO binding to the reduced heme groups can be followed.

Abbreviations used: e^-_{aq}, hydrated electron; IHP, inositol hexaphosphate; bis-tris, 2,2'-bis(hydroxymethyl)-2,2',2"-nitrilotriethanol; CO, carbon monoxide.

Partial reduction of methemoglobin by hydrated electrons produces a number of intermediates, the concentrations of which can be calculated assuming that e^-_{aq} reacts randomly with the ferric heme groups.

An other aspect in the investigation of the kinetics observed for the CO binding to partially reduced methemoglobin is in that the quaternary structure of methemoglobin can be altered by addition of IHP (3).

Experimental

Materials. Human hemoglobin was isolated according to Drabkin (4). After extensive dialysis against destilled water the hemoglobin solutions were freed from organic phosphates by passage through a mixed bed ion-exchange column (Amberlite IRA 400 and IR 120). Methemoglobin was prepared by adding 50% excess of $K_3Fe(CN)_6$ to a solution of oxyhemoglobin. The excess of $K_3Fe(CN)_6$ was removed on a G-25 Sephadex column or by dialysis, followed by passage through a mixed bed ion exchange column.

Horse heart metmyoglobin (Sigma) was used without further purification.

Pulse radiolysis. The irradiation was achieved by a 2 MV Van de Graaf accelerator (High Voltage Engineering Europe), using pulse lengths of 0.5 or 5 µs with a maximum current of 1 A. To ensure homogeneous irradiation of the sample a cell with dimensions of 9 x 5 x 1 mm (optical pathway 9 mm, electron pathway 1 mm) was used. The optical detection system consisted of a xenon arc (XBO 450 W/1, Osram), two Bausch and Lomb grating monochromators (1350 grooves/mm) and a RCA 1P28 photomultiplier; one monochromator was placed between the light source and the cell, the other one between the cell and the detector. The photomultiplier signal was recorded by means of a 7904 Tektronix oscilloscope.

To avoid denaturation of the protein during the removal of oxygen from the solutions the following procedure was used. The buffers were freed from oxygen by passing pure argon through the solutions for 1 h. A concentrated protein solution was deoxygenated in a rotating tonometer by passing argon over it for 15 minutes. A known volume of the concentrated protein solution was transferred anaerobically to the buffer solution. The protein solution was subsequently equilibrated with a mixture of argon and CO. During the experiments a constant flow of this gas mixture was passed over the solution. The mixture was obtained from a gas mixing pump (Wösthoff M300/a-F). The concentration of carbon monoxide in the solutions was calculated using a solubility coefficient of 1.36 µM/mm Hg (1). The concentrations of methemoglobin and metmyoglobin were determined spectrophotometrically. The protein concentrations are given on heme basis.
Static difference spectra were recorded on a Cary 118 spectrophotometer.

As radical scavenger methanol was added up to a concentration of 0.1 M. All kinetic experiments were carried out at room temperature (22 $\pm$ 1°C). Pseudo-first order conditions were satisfied in all experiments.

In cases where a biphasic behavior for the CO binding kinetics was observed, the data were analyzed according to the equation:

$$F(t) = \alpha \exp(-k_1.[CO].t) + (1-\alpha)\exp(-k_2.[CO].t)$$

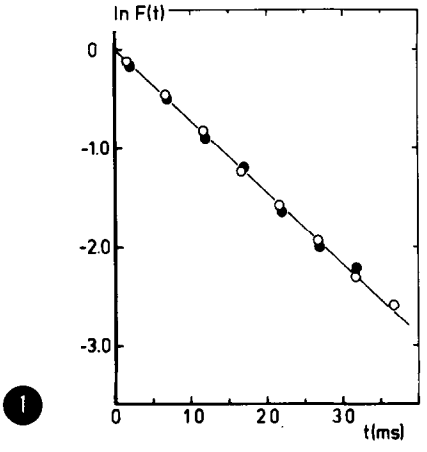
where $F(t) = (A_\infty - A_t)/(A_\infty - A_o)$; A_∞, A_t and A_o being the absorbances at the end of the reaction, at time t and at the beginning of the reaction respectively; k_1 and k_2 the CO binding rate constants; α the fractional contribution of the fast phase to the change in absorbance.

RESULTS AND DISCUSSION

It has been shown before that ferrous hemoglobin obtained by reduction

of methemoglobin by means of e^-_{aq} shows the same functional properties as normal hemoglobin (2). The finding that irradiation does not influence the functional properties of the protein is supported by the kinetics observed for the CO binding to horse heart deoxymyoglobin, produced by the reaction of e^-_{aq} with metmyoglobin. The first order plot shown in fig. 1 demonstrates clearly that the CO binding follows pseudo-first order kinetics with a rate constant of $(4.5 \pm 0.5) \times 10^5 M^{-1} s^{-1}$. This value is in good agreement with the results obtained with stopped flow and flash photolysis experiments (5).

The kinetic difference spectrum for the CO binding to reduced heme groups of human methemoglobin is shown in fig. 2 together with the static difference spectrum of carboxy and deoxyhemoglobin. The figure shows that no significant differences are observed.



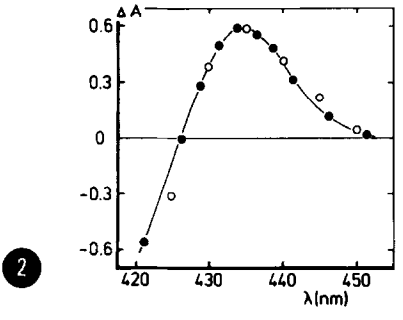


Fig. 1. First order plots for the reaction of CO with myoglobin followed at 435 (o) and 450 (•) nm, after the reduction of metmyoglobin by hydrated electrons. 10 μM metmyoglobin, degree of reduction 0.40; 155μM CO; 5 mM bis-tris, pH 7.0; 0.1 M. methanol; 22°C.

Fig. 2. Kinetic difference spectrum for the reaction of partially reduced methemoglobin with CO (o). Static difference spectrum between deoxy and carboxy hemoglobin (•); 20 μM methemoglobin, degree of reduction 0.34; 25 mM bis-tris, pH 7.0; 0.1 M methanol; 22°C.

The kinetics of the CO binding to partially reduced methemoglobin are strongly dependent on the degree of reduction. Fig. 3 shows the first order plot for the binding of CO to hemoglobin at two degrees of reduction. At a low degree of reduction, i.e. under conditions where the predominant reaction

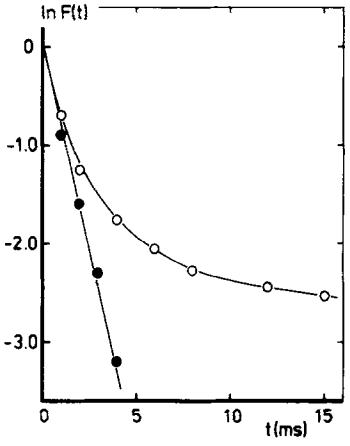


Fig. 3. First order plots for the reaction of CO with partially reduced methemoglobin at a degree of reduction of 0.01 (●) and 0.08 (○). 100 μM methemoglobin; 143 μM CO; 25 mM bis-tris, pH 7.0; 0.1 M methanol; 22°C.

product is a methemoglobin molecule with one reduced heme group, CO binding is monophasic and fast with a rate constant of $(7 \pm 1) \times 10^6 M^{-1} s^{-1}$. Assuming that e^-_{aq} reacts at random with the heme groups of methemoglobin this result indicates the absence of chain heterogeneity with respect to the CO binding. Furthermore, the fast CO binding suggests that a methemoglobin molecule with one reduced heme group is in the R state or the R to T transition is too slow to interfere with the CO binding. This conclusion is not in agreement with an earlier report (2) where the faster of the secondary processes following the reduction of methemoglobin by e^-_{aq} has been assigned to a change in quaternary structure. Recently (unpublished results) this process has been observed under solvent conditions (in 2 M KCl) where methemoglobin is largely dissociated into dimers (6), invalidating the assigment mentioned above.

At higher degrees of reduction where the concentration of molecules with two reduced heme groups becomes significant, a slower phase in the CO binding is observed. In this case the first order plot can be fitted using two exponentials with $k_1 = (7 \pm 1) \times 10^6 M^{-1} s^{-1}$ and $k_2 = (3 \pm 0.5) \times 10^5 M^{-1} s^{-1}$.

The values for the rate constants are within the experimental accuracy independent of the degree of reduction, CO concentration and protein concentration and agree rather well with values observed for fast and slow reacting forms of hemoglobin (1).

In fig. 4 the fractional contribution of the slow phase to the change in absorbance is shown as a function of the degree of reduction. The line in

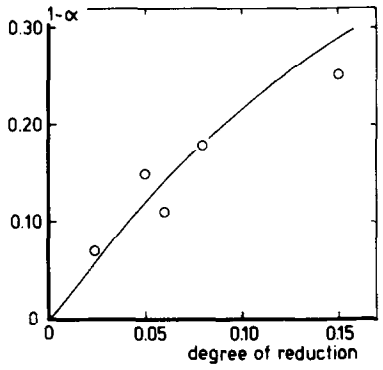


Fig. 4. Fraction slow reacting material found in the reaction of CO with partially reduced methemoglobin as a function of the degree of reduction. The solid line is a theoretical curve representing the fractional contribution of molecules with two reduced heme groups to the change in absorbance. The curve has been calculated assuming that the reduction proceeds at random and that the dissociation constant for methemoglobin has a value of 1 μM. 100 μM methemoglobin; 25 mM bis-tris, pH 7.0; 0.1 M methanol; 22°C.

fig. 4 gives the contribution to the change in absorbance of molecules with two reduced heme groups calculated under the assumption that the reduction of the ferric heme groups by e^-_{aq} proceeds at random and that the dissociation constant for the tetramer-dimer equilibrium for methemoglobin has a value of 1 μM. It can be seen that the experimental points are in reasonable agreement with the calculated curve. This strongly suggests that a hemoglobin tetramer with two ferrous and two ferric heme groups has the T quaternary structure and that under the experimental conditions used the R to T transition for this intermediate is too fast to interfere with the CO binding. This finding is in contrast with the results of Cassoly and Gibson (7) who did not find fast exchange between a slow and fast reacting species observed in the reaction of CO with cyanomet hybrids.

Fig. 5 shows the effect of the presence of 1 mM IHP on the CO binding kinetics. A slow biphasic CO binding is observed independent of the degree of reduction. The first order plots can be fitted using two exponentials: $k_1 = (4.2 \pm 1.8) \times 10^5 M^{-1} s^{-1}$ and $k_2 = (1.2 \pm 0.3) \times 10^5 M^{-1} s^{-1}$. The contribution from each phase is approximately 50% ($\alpha = 0.47 \pm 0.08$). This equality in amplitude of the two phases suggests IHP induced chain heterogeneity as a possible explanation. The fact that in the presence of IHP the CO binding characteristics do not show any dependence on the degree of reduction indicates that as far as the kinetics of the CO binding are concerned methemoglobin

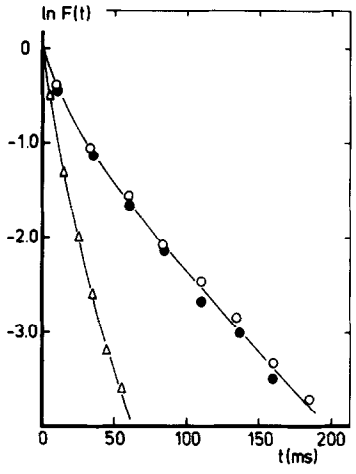


Fig. 5. First order plots for the reaction of CO with partially reduced methemoglobin in the presence of 1 mM IHP: 100 μM methemoglobin, 143 μM CO, degree of reduction 0.02 (•) and 0.07 (o); 100 μM hemoglobin, 450 μM CO, degree of reduction 0.14 (Δ); 25 mM bis-tris, pH 7.0; 0.1 M methanol, 22°C.

tetramers with one or two reduced heme groups are in the same conformational state.

The rate constants found for the two phases differ slightly from those observed for deoxyhemoglobin in the presence of IHP (8). This difference is in accordance with the results of Hensley et al. (9,10), who have observed differences in T structure between deoxyhemoglobin and methemoglobin in the presence of IHP.

In the presence of IHP no influence is found of the protein concentration on the CO binding kinetics. This is in agreement with the fact that in the presence of IHP the dissociation of methemoglobin into dimers is greatly suppressed (11,12).

In conclusion we can say that the pulse radiolysis technique offers a new versatile method for studying ligand binding kinetics to hemoglobin with submillisecond time resolution. Preliminary experiments have shown that this method is also applicable to the study of oxygen binding kinetics to partially reduced methemoglobin.

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