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Communication

Hybrid Formation for Liganded Hemoglobins A and C at Subzero Temperatures*

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Michele Perrella, Michele Samaja, and Luigi Rossi-Bernardi

From the Cattedra di Enzimologia, University of Milan, Via G. Celoria, 2, 20133 Milan, Italy

The kinetics of formation of the asymmetric carbonmonoxyhemoglobin hybrid $(\alpha\beta)^A(\alpha\beta)^C$ from the parent molecules $\alpha_2\beta_2{}^A$ and $\alpha_2\beta_2{}^C$ have been studied by electrophoresis at subzero temperatures (down to −40°C) using as supporting media gels of acrylamide/methylacrylate in dimethyl sulfoxide/water mixtures. It has been found that in these media the rate of hybrid formation is markedly affected by pH and decreases by an order of magnitude between pH 7.3 and 8.3. At pH >10, $t = -40$°C, the hybrid between $\alpha_2\beta_2{}^A$ and $\alpha_2\beta_2{}^C$ is stable for several hours. A rapid thermal quenching of a mixture of $\alpha_2\beta_2{}^A$ and $\alpha_2\beta_2{}^C$ prevented hybrid formation during the time required to separate the 2 molecules.

Isoelectric focusing in polyacrylamide gels has been extensively used (1–3) to study a variety of asymmetrical hemoglobin hybrids, which are formed when two different hemoglobins of unlike charge are mixed together. Since the isoelectric focusing technique is intrinsically slow, all previous studies on this problem have been confined mainly to unliganded hemoglobin, whose dimerization rate is orders of magnitude slower than that of liganded hemoglobin.

We have recently reported (4) that electrophoretic or isoelectric focusing separations can be achieved at subzero temperatures (−30 to −40°C) by using as supporting media gels of acrylamide/methylacrylate copolymers and dimethyl sulfoxide/water mixtures, and we have preliminarily shown that, at low temperatures, hybrid species, which are formed upon mixing human carbonmonoxyhemoglobins A and S, are stable enough to allow their isolation by this technique. More detailed studies on this problem may not only shed further light on the dimerization reaction of liganded hemoglobin, but also promise to indicate appropriate experimental conditions for the isolation of intermediates in the reaction of hemoglobin with oxygen or other ligands. In fact, it has been shown (5) that the "off" reaction of oxygen or carbon monoxide from hemoglobin is considerably slowed down at low temperatures. The stabilization of intermediate compounds of hemoglobin with ligands by a thermal quenching approach may lead to their isolation, if such intermediates do indeed exist in non-vanishing concentration, and if suitable methods of separation at low temperature can be used.

Bunn and McDonough (3) and Park (2) have tried to detect intermediate compounds in the reaction of carbon monoxide with hemoglobin by focusing samples of human hemoglobin partially saturated with carbon monoxide or nitric oxide, but they observed only the separation of fully liganded from fully unliganded hemoglobin, which was possible because of the charge difference due to the Bohr effect. The conditions used in their experiments, however, did not exclude the possibility of ligand exchange reactions between tetramers during the separation process, either by direct ligand reaction exchange or, indirectly, by the dimerization and reassociation reactions of fully or partly liganded hemoglobin.

In this paper, we report studies on the effect of temperature on the rate of dimerization of human carbonmonoxyhemoglobin. Detailed information on this reaction is, indeed, a prerequisite to any attempt to isolate intermediates in the reaction of hemoglobin with ligands, such as carbon monoxide or oxygen, by physical separation methods.

EXPERIMENTAL PROCEDURES

Hemoglobin A_0 and hemoglobin C were obtained from blood samples from a normal and an A-C heterozygote donor and were purified by ion exchange chromatography (6). The hemoglobin used in this work, unless otherwise stated, has been stored and used in the carbonmonoxy form. Electrophoresis at −40°C was carried out as described by Perrella *et al.* (4). The gel-containing tubes were refrigerated by the circulation of a coolant thermostatted at −43°C. The gel temperature during the separation was −40 ± 1°C. Gels were prepared by copolymerization of acrylamide and methylacrylate in a 1:1 molar ratio, $T\% = 8.44$, and methylenebisacrylamide as the cross-linking agent, $C\% = 1.82$ (4).

Gel and electrodic buffers contained 50% (v/v) Me_2SO.[1] The gel buffer was 43 mM Tris and 7.6 mM HCl, and the electrodic buffer was 68.5 mM glycine and 8.6 mM Tris. Their respective pH values at −40°C were approximately 10.5 ± 0.3 and 10.3 ± 0.3. These pH values were obtained by the method of the dye indicators described by Douzou (7). The reported values are approximate because they were extrapolated from pH data measured at 0, −10, −20, and −30°C. It should be added that these buffers are 4 to 7 times more concentrated than those studied by Douzou.

Samples of HbA_0 and HbC of identical concentrations (about 0.4 mM in heme) were incubated as follows. Each sample of HbA_0 or HbC was prepared in aqueous 20 mM phosphate or Tris buffer (7). Solid sucrose was added to each hemoglobin solution to a final 10% (w/v) concentration to increase the viscosity and density of the solution. The samples of HbA_0 or HbC were then introduced into two separate wells at the bottom of a thermostatted glass vessel. When the temperature of the two samples reached 0°C, an equal volume of cold (0°C) ethylene glycol was added, and the temperature of the solutions was then brought to the required value. Mixing was obtained by manually shaking the vessel for 1 or 2 min. Incubation times were considered to start upon shaking the vessel and terminated upon delivery of the samples into the separation cell at −40°C.

Sample delivery was carried out by the use of a microsyringe tipped with a Teflon capillary tube of about 10-μl capacity, which was precooled at the temperature of separation. Twenty to twenty-five micrograms of protein were loaded on each gel tube. Electrophoresis was carried out for 2 to 2½ h at a constant power of about 0.05 watt/gel and 850 V. At the end of the separation, the gel-containing tubes were removed from the cell and microphotographed. Scans of photographic prints were obtained with a Zeiss MQ3 densitometer. Areas under the peaks were integrated by planimetry. Pictures in which the

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[1] The abbreviations used are: Me_2SO, dimethyl sulfoxide; EGOH, ethylene glycol; HbA_0, $\alpha_2\beta_2{}^A$, hemoglobin A_0, CO form; HBC, $\alpha_2\beta_2{}^C$, hemoglobin C, CO form; HbS, hemoglobin S, CO form; $(\alpha\beta)^A(\alpha\beta)^C$, the hybrid hemoglobin species resulting from the dimerization and recombination of a dimer from HbA_0 and a dimer from HbC.

ratio of peak areas corresponding to HbA_0 and HbC was found to exceed the range 1 ± 0.1 were discarded, since the two hemoglobins were used in equimolar amounts.

RESULTS AND DISCUSSION

The amino acid substitution present in HbC ($\beta^{6\ Glu \rightarrow Lys}$) makes the mixture of HbA_0 and HbC used in this work well suited for the electrophoretic separation of the hybrid species from the parent molecules, since it allows a larger charge difference among the three species than that possible with other systems, such as a mixture of HbA_0 and hemoglobin S (4). Since HbA_0 and HbC have practically identical oxygen binding and other functional properties and the amino acid substitution involves a residue located on the surface of the molecule, it can be assumed, in accordance with Bunn and McDonough (3), that the rate constant for the dimerization reaction is similar, if not the same, for the two parent molecules and the hybrid molecule, and that HbA_0, HbC, and their hybrid species have identical thermodynamic stability.

It has been shown (3) that, following the above assumptions, the first order constant for the hemoglobin tetramer dissociation, k, can be calculated from the slope of a plot of $-\ln(1 - 2Y_H)$ *versus* time, where Y_H is the fraction of hybrid species present in an equimolar mixture of HbA_0 and HbC at the incubation time t.

Fig. 1*a* is a photograph of a gel showing the electrophoretic separation at −40°C of an equimolar mixture of HbA_0 and HbC, both in the carbonmonoxy form. The mixture was incubated for 15 min at 0°C in 10 mM phosphate, 50% (v/v) EGOH, pH 7.2. Preliminary experiments have shown that, during this incubation period, the formation of the hybrid species, $(\alpha\beta)^A(\alpha\beta)^C$, practically goes to equilibrium. The experimental conditions used for the electrophoretic separation of the three species, $\alpha_2\beta_2{}^A$, $\alpha_2\beta_2{}^C$, and $(\alpha\beta)^A(\alpha\beta)^C$, *e.g.* T = −40°C, pH > 10, time approximately 2 h, are likely to practically stop dimer exchange between the three species. This is proved by the fact that the middle band, $(\alpha\beta)^A(\alpha\beta)^C$, corresponds to half (±4%) the total hemoglobin, a finding which is expected, on statistical grounds, if complete equilibrium between the three species had been reached during the incubation time and if no appreciable dissociation of the hybrid species had occurred during the electrophoretic separation at −40°C. This situation is confirmed by the electrophoretic separations reported in Fig. 1, *b* and *c*.

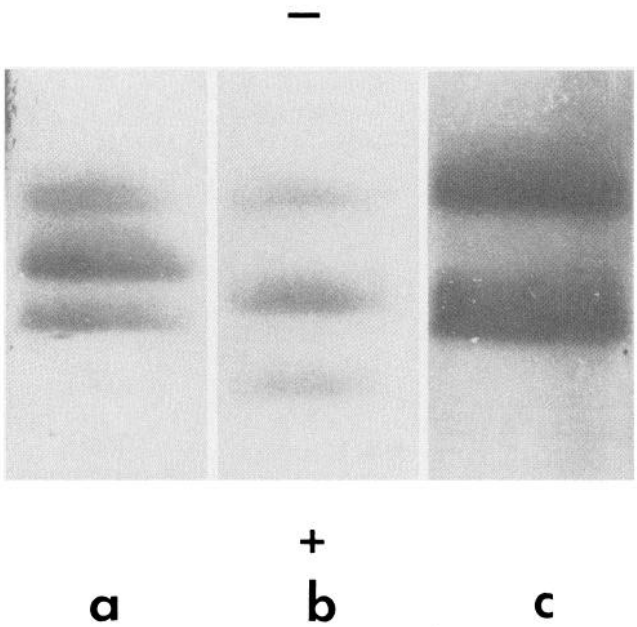

FIG. 1. **Electrophoretic separation.** *a*, electrophoretic separation of an equimolar mixture of carbonmonoxy HbA_0 and HbC, incubated at 0°C for 15 min and then separated at −40°C. *b*, same as *a*, but after separation in three bands the sample was left in the gel for another 2 h, followed by an additional 2-h period of separation. *c*, electrophoretic separation of two samples of HbA_0 and HbC, mixed at −30°C and then separated at −40°C in about 2 h.

In the experiment reported in Fig. 1*b*, electrophoresis of a sample of HbA_0 and HbC was carried out under the same experimental conditions as described for Fig. 1*a*. After separation in three bands, the sample was left in the gel for another 2 h, followed by an additional 2-h period of separation. No decrease in the relative proportion of the middle band was found, indicating that during the equilibration period of 2 h in the gel at −40°C no appreciable formation of $\alpha_2\beta_2{}^A$ and $\alpha_2\beta_2{}^C$ from $(\alpha\beta)^A(\alpha\beta)^C$ has occurred.

The experiment reported in Fig. 1*c* confirms the conclusion reached above. In this case, two samples of HbA_0 and HbC, 10 mM in phosphate, 50% (v/v) EGOH, pH 7.5, were mixed (about 2 min) at −30°C in the thermostatted glass vessel and then separated by electrophoresis at −40°C, pH > 10, in about 2 h. No hybrid species is apparent. This lack of a middle hybrid species was found even if the mixture was left on top of the gel for 1 h. Only increasing the equilibration time at −40°C to 2 h caused the appearance of a very faint middle band. It should be noted that the time required for the sample to enter the gel, and thus to change its pH from about 7.5 to over 10, is, at −40°C, about 30 to 45 min.

Fig. 2 shows logarithmic plots of $(1 - 2Y_H)$ for samples containing equimolar concentrations of HbA_0 and HbC mixed at −10°C and left for various incubation times (abscissa). Fig. 2*a* refers to a sample in 10 mM phosphate, 50% (v/v) EGOH, pH 7.3, whereas Fig. 2*b* refers to identical conditions except for the pH of the incubating mixture, which was = 8.3. Similar

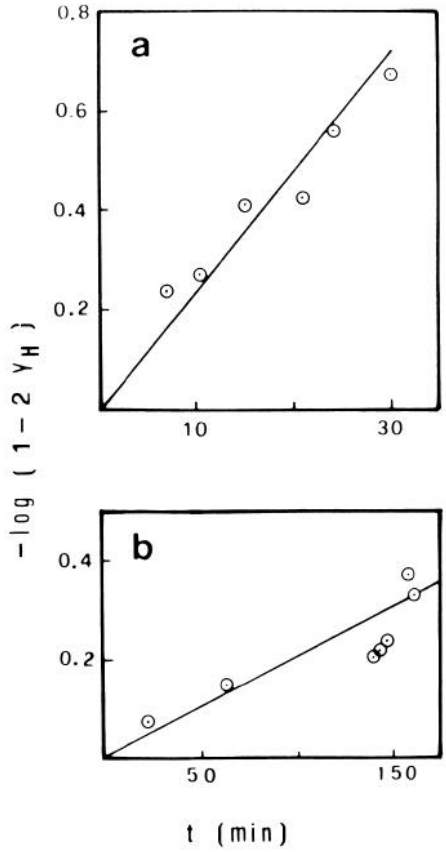

FIG. 2. **Logarithmic plots of $(1 - 2Y_H)$ for samples containing equimolar concentrations of HbA_0 and HbC, mixed at −10°C and left for various incubation times.** *a*, pH 7.3; *b*, pH 8.3.

TABLE I

Values of the apparent velocity constant, k, for the formation of the hybrid $(\alpha\beta)^A(\alpha\beta)^C$ from carbonmonoxy $\alpha_2\beta_2{}^A$ and $\alpha_2\beta_2{}^C$

pH	T	k (min^{-1}) × 10^3
	°C	
7.3	−5	95
7.3	−10	55
7.35	−14	23
7.4	−21	14
7.45	−26	11
8.3	−10	4.7
9.6[a]	−10	1.4

[a] 10 mM Tris-HCl buffer, 50% (v/v) EGOH. All other data were obtained using 10 mM phosphate buffers, 50% (v/v) EGOH.

plots, obtained for experiments in which the temperature of incubation and the pH was varied, allowed the calculation of k, the apparent first order rate constant, for the rate of dissociation of tetramers into dimers (Table I).

The apparent activation energy, E_a, for the dimerization reaction in 10 mM phosphate buffer, pH 7.3, 50% (v/v) EGOH, as calculated from the data of Table I, neglecting the slight pH change of the phosphate buffer over the temperature range considered, is about 13.5 kcal/mol. A marked decrease in the rate constant with increasing pH is observed at subzero temperatures (Fig. 2 and Table I). This effect is in qualitative agreement with the experiments reported by Flamig and Parkhurst (8) for aqueous carboxyhemoglobin at 21.5°C. Presumably, the interpretation of the effect of pH on the decrease in the rate of dimerization given by these authors, *e.g.* formation of new coulombic interactions between charged groups at alkaline pH, also applies under the experimental conditions reported in this paper.

The reader is cautioned against assigning to the values of k, as obtained in this paper, a true physicochemical meaning. In fact, it should be noted that the dielectric constant of 50% (v/v) EGOH in water varies with temperature. Since the forces governing the rate of tetramer dissociation are at least in part electrostatic, it is likely that the absolute value of k will be significantly affected by the presence of EGOH and by the variations in the dielectric constant at various subzero temperatures.

On the other hand, the values of k reported in Table I and the corresponding apparent energy of activation for the dimerization reaction under the solvent conditions used represent useful empirical information for planning experiments for the separation of hybrid species between two different liganded hemoglobins (Fig. 1*a*) or for attempting the demonstration of intermediate compounds of hemoglobin with ligands by this technique. It is relevant to point out that, during the time (about 2 h) required for separation at −40°C (Fig. 1, *a*, *b*, and *c*), no hybrid resulting from the dissociation-association reaction of the two parent tetramers was found. It should be noted that the pH value of the hemoglobin inside the gel is about 10. At this rather alkaline pH and subzero temperature (−40°C), it can safely be assumed that no significant association-dissociation reaction between two different hemoglobin species or between intermediate species (*e.g.* Hb_4X_1, Hb_4X_2, Hb_4X_3, or Hb_4X_4) is likely to occur during the time required to carry out an electrophoretic separation by the technique used.

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