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Isolation of Intermediate Valence Hybrids between Ferrous and Methemoglobin at Subzero Temperatures*

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Michele Perrella, Laura Cremonesi, Louise Benazzi, and Luigi Rossi-Bernardi

From the Cattedra di Enzimologia and Cattedra di Chimica Biologica, University of Milan, Italy

Quenching a hemoglobin solution partially saturated with carbon monoxide into a hydro-organic solvent containing ferricyanide will produce under suitable conditions a population of partially oxidized and CO-bound hemoglobin molecules. Since each Fe^{3+} heme carries one extra charge, it should be possible, in theory, to resolve the spectrum of intermediate compounds between hemoglobin and carbon monoxide, which was originally present in solution. In this study we report: 1) the development of a simple and rapid method to quench aqueous hemoglobin solutions into a hydro-organic solvent at subzero temperatures; 2) the determination of suitable experimental conditions to isolate valence hybrids between carbonmonoxy- and methemoglobin by isoelectric focusing at temperatures as low as −25 °C; and 3) the identification and isolation of all valence hybrids of different charge between carbonmonoxy- and methemoglobin.

If a hemoglobin solution, partially saturated with carbon monoxide or nitric oxide, is rapidly quenched at subzero temperatures into a hydro-organic solvent containing a suitable concentration of ferricyanide, the deoxy hemes will be rapidly oxidized. In order to oxidize the liganded hemes, the ligand has first to dissociate from the iron atom. Douzou (1) and Mosca *et al.* (2) have shown that the rate of dissociation of carbon monoxide from hemoglobin is drastically reduced at subzero temperatures. Thus, it may be feasible to remove the excess ferricyanide before an appreciable amount of CO-bound hemes are oxidized. Since each oxidized Fe^{3+} heme carries one extra charge, it should be possible, using our previously described methods of separation at low temperature (3-5), to resolve the spectrum of intermediate compounds between hemoglobin and carbon monoxide, which was originally present in solution, at equilibrium.

The problem of a physical separation of intermediate compounds of hemoglobin with ligands is, however, more complex. In fact, after the oxidation reaction of the deoxy hemes has been completed, a series of other slower but disturbing reactions will alter with time the pattern of distribution of oxidized hemes between hemoglobin molecules, *i.e.* 1) the association-dissociation reactions between hemoglobin tetramers and dimers, 2) the exchange of hemes, and 3) the transfer of electrons between hemoglobin molecules (6).

In addition, since no data are available as to the stability of partially oxidized hemoglobins in the hydro-organic solvents used at subzero temperatures, a successful approach to the isolation of intermediate compounds of hemoglobin with ligands by electrophoretic methods will also require an extension of Park's (7) pioneer work on the separation of products of partial oxidation of hemoglobin to cover the range of temperature and solvent composition required for such experiments.

The present paper is mainly centered on the work which is preliminary to any attempt to isolate intermediate compounds of hemoglobin with ligands by the approach outlined above. In this study we report (*a*) the development of a simple and rapid method to quench aqueous hemoglobin solutions into an hydro-organic solvent at subzero temperatures; (*b*) the determination of suitable experimental conditions of pH, ionic strength, solvent composition, and temperature under which valence hybrids between carbonmonoxy- and methemoglobin are soluble and stable enough to be isolated by isoelectric focusing at temperatures as low as −25 °C; (*c*) the identification and isolation of all valence hybrids of different charge between carbonmonoxy- and methemoglobin; and (*d*) an estimation of the rate of reactions 1-3 (listed above) at subzero temperature, in hydro-organic solvents.

EXPERIMENTAL PROCEDURES[1]

Preparation of $\alpha_2^+\beta_2^{CO}$ and of $\alpha_2^{CO}\beta_2^+$—Human hemoglobin A_0 and C were purified by ion-exchange chromatography (8) and equilibrated with 0.1 M KCl by gel filtration on Sephadex G-25. Oxyhemoglobin solutions were stored in liquid nitrogen. Carbonmonoxyhemoglobin samples were stored at 4 °C under CO. Methemoglobin solutions were prepared by oxidation of deoxyhemoglobin with three equivalents of ferricyanide at pH 7. Excess ferricyanide was removed by gel filtration on Sephadex G-25 equilibrated with 0.1 M KCl. Methemoglobin was used within a few hours of preparation.

$\alpha_2^+\beta_2^{CO}$ and $\alpha_2^{CO}\beta_2^+$ hybrids were prepared by isoelectric focusing. Samples of oxyhemoglobin (about 70 mg/run), oxidized with a half-equivalent of ferricyanide, were focused on polyacrylamide gel plates (10 × 25 cm) containing 2% (w/v) total concentration of a 1:1 mixture of Ampholine 6-8 and Ampholine 7-9. If hemoglobin and ferricyanide are incubated for 2 h at 25 °C, the hybrid obtained in greater concentration was $\alpha_2^+\beta_2^{O_2}$. Incubation for 5 min at 0 °C yields $\alpha_2^{O_2}\beta_2^+$ predominantly. Following focusing at 7 °C for 2-3 h, a thin slice containing the hybrid of interest was cut out of the gel plate. The hybrid was eluted from the gel in a small volume of 0.1 M KCl for 2-4 h at 0 °C. Concentration was measured by Drabkin's method. The eluted sample was equilibrated with CO and used without removal of Ampholine. Identification of $\alpha_2^+\beta_2^{CO}$ as the component which focuses nearer to the cathode and of $\alpha_2^{CO}\beta_2^+$ as the component which focuses nearer to the anode was obtained by measuring the radioactivity associated with the α and the β chains, after treating each derivative with radioactive cyanide ($^{14}CN^-$). Since heme exchange reactions are slow for HbCN

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[1] The abbreviations used are: $\alpha_2\beta_2$, human hemoglobin A_0; $\alpha_2^{CO}\beta_2^{CO}$, the same saturated with carbon monoxide; in $\alpha_2^+\beta_2^{CO}$ and $\alpha_2^{CO}\beta_2^+$, the superscript + represents oxidized hemes and CO is carbon monoxide-bound hemes; *p*-CMB, parachloromercuribenzoate; EGOH, ethylene glycol; MeOH, methanol; $\alpha_2\beta_2^C$ = human hemoglobin C; $(\alpha\beta)^A(\alpha\beta)^C$, the hybrid hemoglobin species resulting from the dimerization and recombination of a dimer from hemoglobin A_0 and a dimer from hemoglobin C. pa_H represents the hydrogen ion activity in hydro-organic solvents.

(6) and autooxidation also is slow in the presence of carbon monoxide, the radioactivity should be associated only with the Met chains.

The gel slice containing $\alpha_2^+\beta_2^{CO}$ or $\alpha_2^{CO}\beta_2^+$ was suspended for 1 h at 0 °C, under CO, in 400 μl of 0.1 M KCl containing a 20-fold molar excess of $^{14}CN^-$ over heme concentration. The $K^{14}CN$ specific activity was about 0.05 mCi/mmol. The eluate was reacted with a 10-fold molar excess of *p*-CMB under CO at 0 °C for 1 h. The mercuriated α and β chains were separated from the unreacted protein by polyacrylamide gel isoelectric focusing, and the gel slices containing the chains were dissolved in a scintillation mixture for radioactivity measurement. The radioactivity associated with the α chains derived from $\alpha_2^+\beta_2^{CO}$ was at least 10 times higher than that associated with the β chain fraction. The reverse finding was found for $\alpha_2^{CO}\beta_2^+$, thus confirming the identification of the two hybrids.

Preparation of Samples for Subzero Temperature Isoelectric Focusing—The stepwise procedure described by Douzou (1) for preparation of protein samples in hydro-organic solvents, although effective in avoiding denaturation, requires considerable time and thus is not suitable for quenching at low temperatures fairly rapid reactions such as those relating to hemoglobin hybrid formation and heme exchange between partially oxidized hemoglobin molecules. It was thus essential to develop a more rapid procedure for preparation of hemoglobin samples for subzero temperature work which would also allow a rapid thermal quenching of such reactions. The apparatus developed for such a purpose is shown in Fig. 1. It consists of parts obtained from a Radiometer vibrating tonometer (which can be obtained as a spare part from Radiometer, Denmark) to which a new glass part has been fitted. A variety of such glass parts can be easily made by an experienced glass blower to suit anaerobic experiments. In a typical quenching experiment, a mixture of 50% EGOH (v/v) and a 20 mM phosphate buffer (the pH of the phosphate buffer at 25 °C was 7.5) was cooled to −25 °C in the apparatus. The tonometer was then started, and the required amount of hemoglobin sample was directly squirted by a microsyringe into the solution. Complete physical mixing was estimated to occur in less than 1 s, but temperature equilibration may require much less. No precipitate was noted when the final concentration of hemoglobin or of the hybrids $\alpha_2^+\beta_2^{CO}$ and $\alpha_2^{CO}\beta_2^+$ was varied between 10 to 3 g/liter.

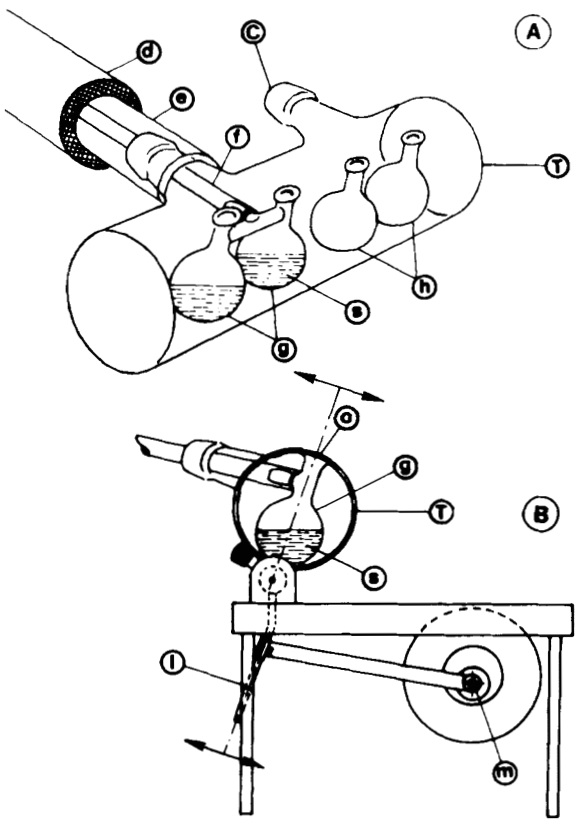

FIG. 1. **Details of a tonometer and the mixing arrangements.** *A*, tonometer suitable for mixing at subzero temperatures under a controlled atmosphere a hydro-organic solvent with a protein solution. *T* is a glass tonometer containing four vessels. Vessels *g* are for work under a controlled atmosphere. Vessels *h* are used for storing solutions. The hydro-organic solvent *s* is shown in *g*. *f* is a silicone tubing used for passing gases over the vessels. Tubing *e* and *d* are used for the cooling fluid and the thermal insulation, respectively. Another tubing (not shown) is connected to *c* for circulating the thermostatting fluid. *B*, details of the mixing arrangement. A motor is connected by the lever *m* to bar *l*, thus providing a shaking mechanism for the tonometer *T* and its contents. For quenching a protein solution into the hydro-organic solvents, the motor is first activated and the protein solution is squirted from a microsyringe into the hydro-organic solvent through opening *o*.

Isoelectric Focusing at Subzero Temperatures—Isoelectric focusing at subzero temperatures on gel tubes 9 cm long was performed as previously described (3). Gels were prepared by co-polymerization of acrylamide with ethylacrylate, using methylenebisacrylamide as the cross-linker (3). The polymer composition was T'% = 7.7, C'% = 1.56 (acrylamide/ethylacrylate molar ratio of 1.5). The cryosolvent was a ternary mixture of EGOH (20%)-MeOH (15%)-H_2O (65%), where the percentage is on a volume basis. This mixture freezes at a temperature close to −30 °C. Methanol was added to the mixture ready for polymerization just prior to filling the glass tubes. Further details for the low temperature focusing technique, including the measurement of pa_H gradients in the gels, can be found in our previously reported studies (3, 5).

Scans of the gels contained in the glass tubes were carried out at 20 °C using a Cliniscan apparatus (Helena Laboratories, Beaumont, TX) at 525 nm with a 2.5 × 0.1-mm slit. At this wavelength, carboxy- and methemoglobin at 20 °C, in the hydro-organic solvent used, are approximately isosbestic.

RESULTS

Migration of Carboxy- and Methemoglobin in pa_H Gradients at Subzero Temperatures—The obvious target in separation experiments at subzero temperatures is the optimization of experimental conditions for reducing the time required for attaining the focusing equilibrium. The rate of migration of hemoglobin dissolved in hydro-organic solvents depends on the following factors: 1) gel temperature and solvent composition. Increase in solvent viscosity at low temperatures will decrease protein mobility. Ternary mixtures of EGOH, MeOH, and water containing more than 15% methanol do have lower viscosity. Unfortunately, we have found that gels containing higher than 15% methanol are not suitable for focusing experiments. In fact, if the ternary mixture EGOH (20%)-MeOH (20%)-H_2O (60%) is used, the gel undergoes structural changes at temperatures below −20 °C, resulting in a slower rate of migration and serious band distortion. The ternary mixture used in this work (see "Experimental Procedures") seems to be the best compromise for operating in the temperature range ≤−27 °C. 2) Distance from the gel top to the equilibrium position. This should be as short as possible in order to decrease the time required for the attainment of equilibrium. This distance is a function of the pa_H of the anolyte and of the catholyte, of the pa_H gradient in the gel and, obviously, of the isoelectric point of the protein under study. 3) Shape of the pa_H gradient. The mobility is low in flat gradients which, on the other hand, allow the best resolution of proteins having close isoelectric points, such as the hemoglobin valency hybrids object of this study.

In practice, a wide range of conditions may be chosen by selecting the proper combination of temperature and pH range of Ampholine used as catholyte and anolyte and in the gel and the composition of the hydro-organic solvent. Fig. 2 shows values of pa_H determined at different points in gels of acrylamide/ethylacrylate co-polymers in 20% (v/v) EGOH-15% (v/v) MeOH-65% (v/v) H_2O at −12 and −23 °C. The pa_H determinations were made as previously described (5). Fig. 2*c* shows that at equilibrium carboxy- and methemoglobin focus about 9 mm apart, 20 to 30 mm from the gel top. The conditions chosen for this experiment thus represent a reasonable compromise between resolution and distance of the equi-

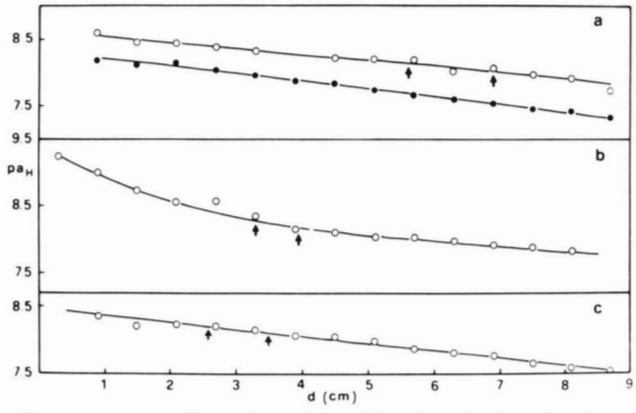

FIG. 2. **pa$_H$ gradients in tube gel isoelectric focusing in 20% (v/v) EGOH-15% (v/v) MeOH-65% (v/v) water at subzero temperatures.** *a*, pa$_H$ gradients at −12 °C. ○, 1% Ampholine, pH 8–9.5, as catholite, 0.1% Ampholine, pH 6–8, as anolite, 3.2% Ampholine, pH 6–8, in the gel. *Arrows* show the positions reached in the gel by methemoglobin and carbonmonoxyhemoglobin after 24-h focusing. ●, 1% Ampholine, pH 7–9, as catholite, 0.1% Ampholine, pH 5–7, as anolite, 3.2% Ampholine, pH 5–7, in the gel. *b*, pa$_H$ gradient at −23 °C. Ampholine ranges as in *a* (○). *c*, pa$_H$ gradient at −23 °C. 1% Ampholine, pH 7–9, as catholite, 0.1% Ampholine, pH 6–8, as anolite, 3.2% Ampholine, pH 6–8, in the gel.

librium position from the gel top. Fig. 2*b* shows how steeper pH gradients in the gel affect the difference in the equilibrium position of carbonmonoxy and methemoglobin.

Fig. 3 reports data on the mobility of carboxyhemoglobin at −12 and at −23 °C, as well as data on the variation with time of the distance of separation between methemoglobin and carboxyhemoglobin.

Separation and Identification of Valence Hybrids of Methemoglobin—It has been previously established (9, 10) that both liganded and methemoglobin tetramers rapidly dissociate into dimers by cleavage along the $\alpha_1\beta_2$ subunit contacts to yield $\alpha_1\beta_1$ dimers. When two different hemoglobins are mixed together, an asymmetrical hemoglobin hybrid may be formed by exchange of dimers between the two different molecules. Since electrophoretic or isoelectric focusing techniques of separation are intrinsically slow, previous attempts to separate hybrids between two different liganded hemoglobins have failed to separate their asymmetrical hybrid (11, 12). We have previously shown that the kinetics of formation of the asymmetric carbonmonoxyhemoglobin hybrid $(\alpha\beta)^A$ $(\alpha\beta)^C$ from the parent molecules $\alpha_2\beta_2$ and $\alpha_2\beta_2^C$ can be studied by electrophoresis at subzero temperatures (down to −40 °C).

The development of the isoelectric focusing technique at subzero temperatures and the availability of the hemoglobin species, α_2^{CO} β_2^{CO}, $\alpha_2^+\beta_2^+$, $\alpha_2^+\beta_2^{CO}$, and α_2^{CO} β_2^+, makes it now possible to carry on a variety of hybridization experiments between any of these four available compounds. The aim of such experiments is to verify the existence, the stability, and the electrophoretic behavior of such valence-hybrid compounds.

Fig. 4 reports an isoelectric focusing pattern obtained by mixing, under various conditions of temperature, time of incubation, and pa$_H$, $\alpha_2^+\beta_2^{CO}$, $\alpha_2^{CO}\beta_2^+$, $\alpha_2^{CO}\beta_2^{CO}$, and $\alpha_2^+\beta_2^+$. Fig. 4*A*, *2*, shows that incubation of such molecules for up to 30 min at −23 °C, pa$_H$ 8.5, followed by separation under the conditions reported in Fig. 2, at −23 °C, does not lead to the formation of appreciable amounts of the $(\alpha^{CO}\beta^{CO})$ $(\alpha^+\beta^+)$ asymmetrical hybrids. On the contrary, such a hybrid is formed and detected either by incubating $\alpha_2^{CO}\beta_2^{CO}$ and $\alpha_2^+\beta_2^+$ for 5 min at 0 °C (Fig. 4*A*, *1*) or for 30 min at −23 °C at pa$_H$ 7.5 (Fig. 4*A*, *3*). It is clear that (1) at pa$_H$ 8.5 and −23 °C the rate of hybrid formation is negligible in comparison with the time required

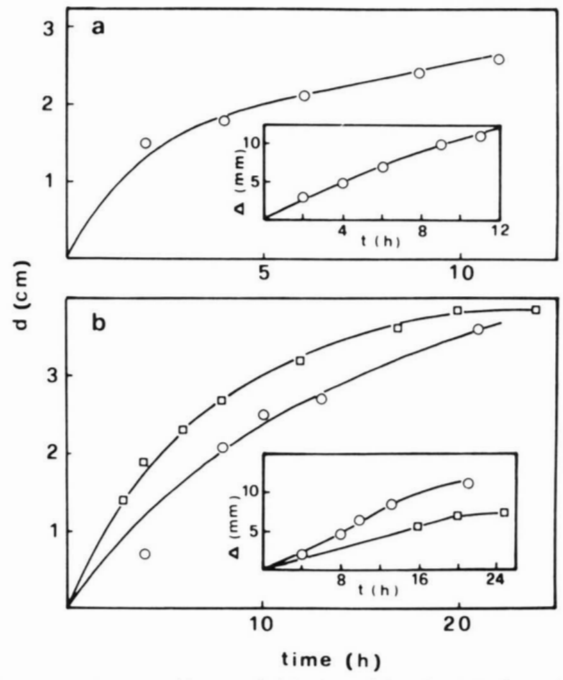

FIG. 3. **Mobility of hemoglobin in gel isoelectric focusing at subzero temperatures.** *a*, mobility of carbonmonoxyhemoglobin at −12 °C in the pa$_H$ gradient of Fig. 2*a* (○). *Inset:* Δ is the distance (mm) between methemoglobin and carbonmonoxyhemoglobin during approach to equilibrium. *b*, □, mobility of carbonmonoxyhemoglobin at −23 °C in the pa$_H$ gradient of Fig. 2*b*; ○, mobility of carbonmonoxyhemoglobin at −23 °C in the pa$_H$ gradient of Fig. 2*c*. *Inset*, same as *a*.

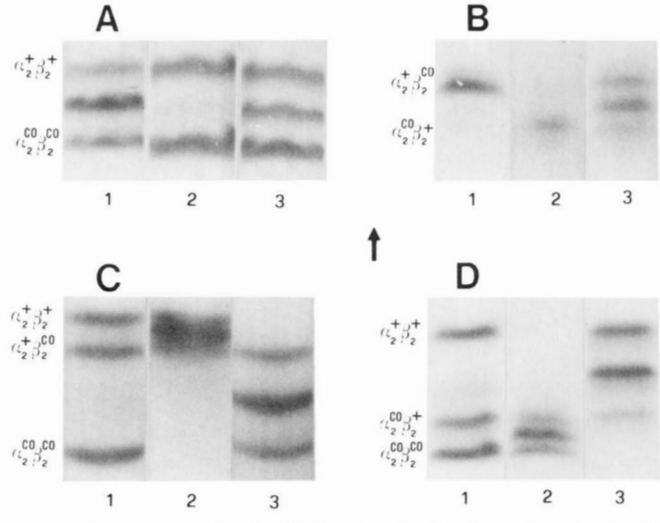

FIG. 4. **Patterns obtained after isoelectric focusing at −23 °C of various hemoglobin species.** The *arrow* indicates the direction of the increasing pa$_H$ in the gradient. *A*, *1*, $\alpha_2^+\beta_2^+$ is mixed and incubated with $\alpha_2^{CO}\beta_2^{CO}$ for 5 min at 0 °C. The mixture is then quenched and separated at −23 °C as described in the text. *2*, $\alpha_2^+\beta_2^+$ is mixed with $\alpha_2^{CO}\beta_2^{CO}$ at −23 °C, pa$_H$ 8.5, and the mixture is incubated for 30 min under these conditions prior to focusing. *3*, the same as *2*, but at pa$_H$ 7.5. *B*, *1*, $\alpha_2^+\beta_2^{CO}$; *2*, $\alpha_2^{CO}\beta_2^+$; *3*, $\alpha_2^+\beta_2^{CO}$ and $\alpha_2^{CO}\beta_2^+$ mixed and incubated at 0 °C for 5 min prior to quenching at −23 °C. *C*, *1*, $\alpha_2^+\beta_2^+$, $\alpha_2^+\beta_2^{CO}$, and $\alpha_2^{CO}\beta_2^{CO}$ mixed and incubated at −23 °C, pa$_H$ 8.5, for 5 min prior to focusing, *2*, $\alpha_2^+\beta_2^+$ and $\alpha_2^+\beta_2^{CO}$ mixed and incubated at 0 °C for 5 min prior to quenching at −23 °C. *3*, $\alpha_2^+\beta_2^{CO}$ and $\alpha_2^{CO}\beta_2^{CO}$ mixed and incubated at 0 °C for 5 min prior to quenching at −23 °C. *D*, *1*, $\alpha_2^+\beta_2^+$, $\alpha_2^{CO}\beta_2^+$, and $\alpha_2^{CO}\beta_2^{CO}$ mixed at −23 °C, pa$_H$ 8.5, and incubated for 5 min under these conditions prior to focusing. *2*, $\alpha_2^{CO}\beta_2^+$ and $\alpha_2^{CO}\beta_2^{CO}$ mixed and incubated at 0 °C for 5 min prior to quenching at −23 °C; *3*, $\alpha_2^{CO}\beta_2^+$ and $\alpha_2^+\beta_2^+$ mixed and incubated at 0 °C for 5 min prior to quenching at −23 °C.

for the isolation of hemoglobin species and (2) that the hybrid $(\alpha^{CO}\beta^{CO})$ $(\alpha^{+}\beta^{+})$, once formed, can be detected by the isoelectric focusing technique employed, being stable enough to live for the period of time required for protein separation.

The experiment reported in Fig. 4 confirms our previously reported data on the isolation of the hybrid between carbonmonoxyhemoglobins A and C. The *middle band* shown in Fig. 4*A*, *1*, corresponds to about half the total hemoglobin, a finding which is expected, on statistical grounds, only if complete equilibrium of the dimer exchange process between $\alpha_2^+\beta_2^+$ and $\alpha_2^{CO}\beta_2^{CO}$ has been reached, and if no appreciable dissociation of the hybrid species $(\alpha^{CO}\beta^{CO})$ $(\alpha^{+}\beta^{+})$ has occurred during the isoelectric focusing separation.

Fig. 4*B* shows a somewhat similar experiment in which $\alpha_2^+\beta_2^{CO}$ and $\alpha_2^{CO}\beta_2^+$ are incubated for 5 min at 0 °C and then separated at −23 °C. The middle band in Fig. 4*B*, *3*, corresponds to the $(\alpha^{+}\beta^{CO})$ $(\alpha^{CO}\beta^{+})$ hybrid species. Again such a hybrid is present with a concentration roughly half the total hemoglobin concentration, which demonstrates its stability under the condition chosen for its separation.

Fig. 4*C* shows that by mixing $\alpha_2^+\beta_2^+$ and $\alpha_2^+\beta_2^{CO}$ and by mixing $\alpha_2^+\beta_2^{CO}$ and $\alpha_2^{CO}\beta_2^{CO}$ at 0 °C another two hybrid species are obtained, which most likely can be identified as $(\alpha^{+}\beta^{+})$ $(\alpha^{+}\beta^{CO})$ (Fig. 4*C*, *2*) and as $(\alpha^{+}\beta^{CO})$ $(\alpha^{CO}\beta^{CO})$ (Fig. 4*C*, *3*). Again, it can be shown that by mixing and incubating the parent species $\alpha_2^+\beta_2^+$, $\alpha_2^+\beta_2^{CO}$, and $\alpha_2^{CO}\beta_2^{CO}$ at −23 °C for 5 min, followed by separation at the same temperature, no hybrid species can be detected (Fig. 4*C*, *1*).

Fig. 4*D* represents the last experiment of this kind, leading to the demonstration of the remaining hybrid species between methemoglobin and carbonmonoxyhemoglobin that can be theoretically detected by the approach outlined in this paper. In this case, mixing $\alpha_2^{CO}\beta_2^+$ and $\alpha_2^{CO}\beta_2^{CO}$ for 5 min at 0 °C and separation at −23 °C lead to the detection of another hybrid species, *i.e.* $(\alpha^{CO}\beta^{+})$ $(\alpha^{CO}\beta^{CO})$ (Fig. 4*D*, *2*), and mixing $\alpha_2^+\beta_2^+$ and $\alpha_2^{CO}\beta_2^+$ yields $(\alpha^{+}\beta^{+})$ $(\alpha^{CO}\beta^{+})$ (Fig. 4*D*, *3*). Again no hybrid formation is obtained when the three parent compounds $\alpha_2^+\beta_2^+$, $\alpha_2^{CO}\beta_2^+$, and $\alpha_2^{CO}\beta_2^{CO}$ are mixed together and separated at −23 °C (Fig. 4*D*, *1*).

It should be pointed out that the incubation time of 5 min at 0 °C was found sufficient to allow the rapid dimer exchange reaction between two unlike hemoglobin species to reach equilibrium, as shown by the statistical distribution between the hybrid and its parent molecules. If this incubation time is increased, secondary reactions such as those described by Bunn and Jandl (6) will take place. These authors have, in fact, shown that intact heme groups undergo exchange between molecules of human hemoglobin under physiological conditions and also produced some evidence of a parallel electron exchange reaction between molecules of different charge. Fig. 5 shows that when $\alpha_2^{CO}\beta_2^{CO}$ and $\alpha_2^+\beta_2^+$ are incubated for up to 4 h at 25 °C and then separated at 2–4 °C by isoelectric focusing in aqueous solvents, the well known pattern of distribution of four bands appears (Fig. 5, *f*) (13). The two middle bands, as described in the first part of this section, are $\alpha_2^+\beta_2^{CO}$ and $\alpha_2^{CO}\beta_2^+$. It is interesting to note that appreciable amounts of such hybrids can only be formed, at 2–4 °C, by incubation of $\alpha_2^{CO}\beta_2^{CO}$ and $\alpha_2^+\beta_2^+$ for some hours, whereas no compounds of this kind are produced during the time (5–10 min) which has been used for the hybridization experiments reported in Fig. 4, *A–D*. No hybrid species resulting from rapid dimer exchange can obviously be traced under the experimental conditions used for the separations reported in Fig. 5, since at 2–4 °C the rate of attainment of the tetramer-dimer equilibrium is faster than the rate of the separation process. However, this is not the case for the symmetrical hybrids $\alpha_2^+\beta_2^{CO}$ and $\alpha_2^{CO}\beta_2^+$, which can only be transformed back into the two species $\alpha_2^+\beta_2^+$ and $\alpha_2^{CO}\beta_2^{CO}$ by heme or electron exchange reactions, which, as Bunn and Jandl (6) have demonstrated, are much slower than both the dimer exchange reactions and the separation process itself.

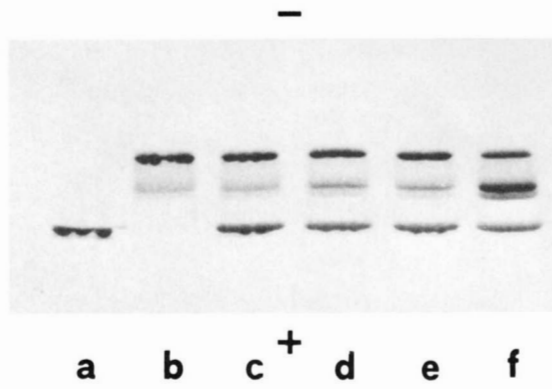

FIG. 5. **The pattern obtained after isoelectric focusing at 4 °C.** *a*, $\alpha_2^{CO}\beta_2^{CO}$; *b*, $\alpha_2^+\beta_2^+$; *c*, $\alpha_2^{CO}\beta_2^{CO}$ mixed and incubated for 10 min at 0 °C with $\alpha_2^+\beta_2^+$; *d*, the same as *c* but with an incubation time of 4 h at 0 °C before focusing; *e*, the same as *c* but with an incubation time of 10 min at 25 °C; *f*, the same as *c* but with an incubation time of 4 h at 25 °C.

DISCUSSION

The rapid oxidation of a partially liganded hemoglobin solution, followed by the removal of the excess oxidant before oxidation of the liganded hemes has taken place, will produce a mixture of partially oxidized hemoglobin molecules. A total of 16 such species (Fig. 6) is theoretically possible. Since each compound of the type 0–IV (Fig. 6) carries a different charge, it should be possible, by appropriate electrophoretic methods, to isolate and quantify the distribution of such species and thus determine the spectrum of intermediate compounds between hemoglobin and ligand, which was originally present, at equilibrium. Dissociation-association reactions between dimers, heme, and/or electron exchange, however, will alter with time the original pattern of distribution of oxidized hemes between various hemoglobin molecules. Since the rate of such reactions is slowed down by a decrease in temperature, as shown by Park in her pioneer work on this subject (7), it was necessary to develop suitable methods to obtain isoelectric focusing and electrophoretic separation at low temperatures. Park's work indicated that the limit to low temperature separations of proteins by focusing could be traced down to irreversible changes in gel structure at temperatures lower than −15 °C. By using as supporting media gels of acrylamide/methylacrylate co-polymers, we have previously shown (3–5) that satisfactory separations of hemoglobin A_0 and hemoglobin C and of their hybrid $(\alpha\beta)^A(\alpha\beta)^C$ can be obtained at temperatures as low as −40 °C.

The work reported in this paper extends such studies to the isolation and characterization of the different valence hybrid compounds between carbonmonooxyhemoglobin and methemoglobin. The data on the mobility and distance of separation between charged hemoglobin molecules, dissolved in hydroorganic solvents, together with the details reported under "Materials and Methods," give the essential information for achieving satisfactory separations of the different valence hybrids of hemoglobin at various subzero temperatures.

Since four "stable" species, *i.e.* $\alpha_2^{CO}\beta_2^{CO}$, $\alpha_2^+\beta_2^+$, $\alpha_2^+\beta_2^{CO}$, and $\alpha_2^{CO}\beta_2^+$, can be readily prepared, a variety of hybridization experiments can be performed, yielding a qualitative estimation of the stability with time of various valence hybrid species. Fig. 5 shows that reactions such as heme or electron exchange are intrinsically slow, as first demonstrated by Bunn

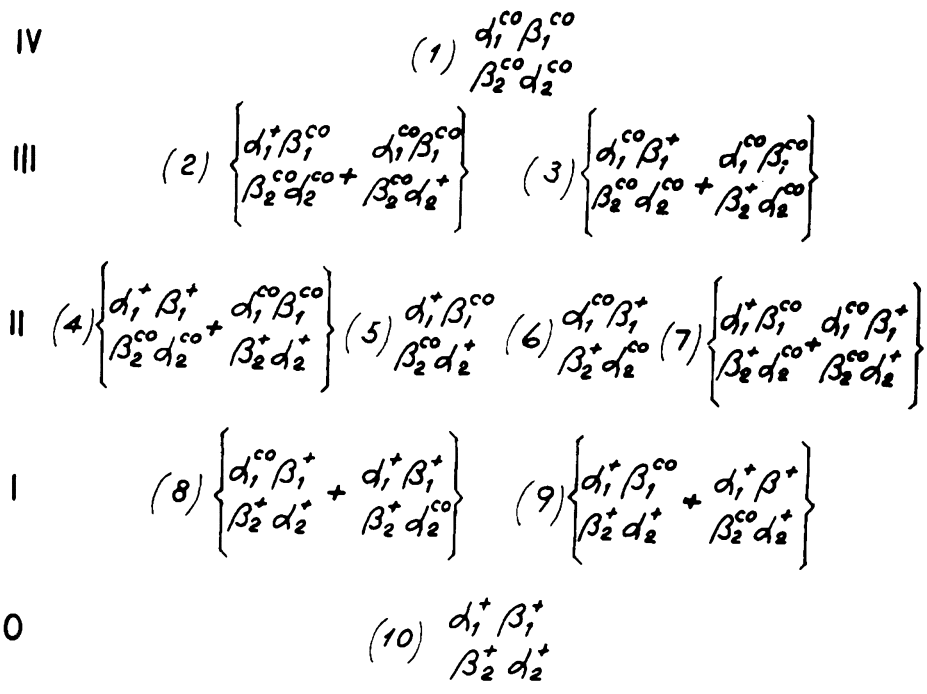

FIG. 6. **Valence hybrids theoretically obtainable by partial oxidation of $\alpha_2^{CO}\beta_2^{CO}$.** Hybrids shown in *brackets* are equivalent.

and Jandl. In fact, appreciable amounts of $\alpha_2^{CO}\beta_2^{+}$ and $\alpha_2^{+}\beta_2^{CO}$ can only be obtained by incubating $\alpha_2^{CO}\beta_2^{CO}$ and $\alpha_2^{+}\beta_2^{+}$ for several hours at 2–4 °C. It should be pointed out that $\alpha_2^{+}\beta_2^{CO}$ and $\alpha_2^{CO}\beta_2^{+}$ can only be formed from $\alpha_2^{CO}\beta_2^{CO}$ and $\alpha_2^{+}\beta_2^{+}$ by heme or electron exchange reactions since it has been previously found (9, 10) that liganded or methemoglobin cleave along the same $\alpha_1\beta_2$ interface to yield $\alpha_1\beta_1$ dimers. Thus, association-dissociation reactions into dimers of $\alpha_2^{CO}\beta_2^{CO}$ and $\alpha_2^{+}\beta_2^{+}$ may only yield a hybrid of the $(\alpha^{+}\beta^{+})$ $(\alpha^{CO}\beta^{CO})$ type.

Fig. 4*A*, *2*, shows that, at −23 °C, $pa_H = 8.5$, no appreciable amount of the hybrid $(\alpha^{+}\beta^{+})$ $(\alpha^{CO}\beta^{CO})$ is formed when $\alpha_2^{CO}\beta_2^{CO}$ and $\alpha_2^{+}\beta_2^{+}$ are mixed and incubated together for up to 30 min and then separated by isoelectric focusing at low temperature. Clearly, under the experimental conditions chosen, the rate of tetramer dissociation of $\alpha_2^{CO}\beta_2^{CO}$ and/or $\alpha_2^{+}\beta_2^{+}$ is very slow. This, furthermore, is confirmed by the results shown in Fig. 4*A*, *1*. In this case, it was found that the distribution, at equilibrium, between $\alpha_2^{CO}\beta_2^{CO}$, $(\alpha^{CO}\beta^{CO})$ $(\alpha^{+}\beta^{+})$, and $\alpha_2^{+}\beta_2^{+}$ was close, to within ±5%, to the theoretical statistical value of 1:2:1. This situation would not be found if the rate of dissociation of the hybrid was not negligible.

We have previously estimated (4) that k (or b in the terminology used by Park in Ref. 7, the apparent first order constant for the rate of tetramer dissociation into dimers) is much influenced by a decrease in temperature, since the apparent activation energy E_a for this reaction was estimated to be ~13.5 kcal/mol. By extrapolation of our previous data (4) a value of $k = 1.4 \times 10^{-3}$ min^{-1} is obtained for the rate of dissociation at −23 °C, $pa_H = 8.5$, of HbA and/or HbC dissolved in a 10 mM phosphate buffer in 50% (v/v) EGOH. By means of a procedure similar to that outlined in our previous study (*i.e.* determination of the hybrid species $(\alpha^{+}\beta^{+})$ $(\alpha^{CO}\beta^{CO})$ after incubating $\alpha_2^{CO}\beta_2^{CO}$ and $\alpha_2^{+}\beta_2^{+}$ for various periods of time in a 10 mM phosphate buffer containing 20% (v/v) EGOH and 15% (v/v) MeOH, $pa_H = 8.5$), a value of $k = 1.5 \times 10^{-3}$ min^{-1} was obtained. A higher value, $k = 2.6 \times 10^{-3}$ min^{-1} was estimated if the phosphate concentration was raised to 20 mM, indicating that the ionic strength is also important in determining the hybrid stability.

In order to estimate the value of k under the conditions met by the various hemoglobin species during the separation in the gel, mixtures of $\alpha_2^{+}\beta_2^{+}$, $\alpha_2^{CO}\beta_2^{CO}$ and of $(\alpha^{+}\beta^{+})$ $(\alpha^{CO}\beta^{CO})$, which were prepared as described in Fig. 4*A*, *1*, were focused for up to 20 h, after the attainment of the equilibrium in the pa_H gradient, together with a sample of HbA_2 in the carbonmonoxy form.

By determining the decrease with time of the ratio between the concentration of the hybrid $(\alpha^{+}\beta^{+})$ $(\alpha^{CO}\beta^{CO})$ with respect to the concentration of HbA_2, used as a reference, the rate of hybrid dissociation could be estimated for the conditions of ionic strength prevailing in the gel during focusing. A value of $k = 0.6 \times 10^{-3}$ min^{-1} was obtained from such measurements, indicating that the dissociation of hybrid $(\alpha^{+}\beta^{+})$ $(\alpha^{CO}\beta^{CO})$ has a half-time in excess of 60 h. All such findings thus indicate that both the rate of tetramer-dimer exchange as well as the rate of heme and/or electron exchange are negligible, under the conditions of temperature and pa_H chosen for the electrophoretic separation of $\alpha_2^{CO}\beta_2^{CO}$, $\alpha_2^{+}\beta_2^{+}$ and of their valence hybrid.

The data reported in Fig. 4, *B* and *D*, do indeed show that similar values of k, at least qualitatively, will most likely be met for the rate of dissociation of the other valence hybrids shown in Fig. 6.

Again, for all species investigated there was no evidence of hybrid formation if any two of the four species $\alpha_2^{CO}\beta_2^{CO}$, $\alpha_2^{+}\beta_2^{+}$, $\alpha_2^{CO}\beta_2^{+}$, and $\alpha_2^{+}\beta_2^{CO}$ were mixed and incubated for up to 30 min at −23 °C, pa_H 8.5, and then separated at the same temperature and similar pa_H value (Fig. 4*C*, *1*, and Fig. 4*D*, *1*). In addition, any of the hybrid formed by incubating at higher temperatures any two of the four stable species was found to have a concentration close to the theoretical statistical ratio 1:2:1 with respect to its parent molecules (Fig. 4*B*, *3*, Fig. 4*C*, *2* and *3*, Fig. 4*D*, *2* and *3*). Such finding analogous to that found for $\alpha_2^{CO}\beta_2^{CO}$, $\alpha_2^{+}\beta_2^{+}$ and their hybrid $(\alpha^{+}\beta^{+})$ $(\alpha^{CO}\beta^{CO})$ do indeed indicate that the rate of the separation process is much faster than the rate of dissociation of the hybrid species.

With reference to Fig. 6, it is now clear that all compounds of the types 0–IV have been isolated in the various hybridization experiments. Compounds 1, 5, 6, and 10 represent the four stable species. Species 7 should correspond to the *middle band* shown in Fig. 4*B*, *3*, species 9 to the *middle band* shown in Fig. 4*C*, *2*, species 2 to the *middle band* of Fig. 4*C*, *3*, species 3 to the *middle band* shown in Fig. 4*D*, *2*, and finally species 8 to the *middle band* of Fig. 4*D*, *3*.

We believe that the results reported in this paper may bring us nearer to the physical isolation of the spectrum of intermediate compounds between hemoglobin and its ligands if such compounds do indeed exist in nonvanishing amounts.

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