Hemoglobin is the paradigm of allosteric proteins. Over the years, cooperative oxygen binding has been explained by different models predicting that the T state of hemoglobin binds oxygen either noncooperatively or with some degree of cooperativity or with strong cooperativity. Therefore, a critical test that discriminates among models is to determine the oxygen binding by the T state of hemoglobin. Fixation of hemoglobin in the T state has been achieved either by crystallization from polyethylene glycol solutions or by encapsulation in wet porous silica gels. Hemoglobin crystals bind oxygen noncooperatively with reduced affinity compared with solution, with no Bohr effect and with no influence of other allosteric effectors. In this study, we have determined accurate oxygen-binding curves to the T state of hemoglobin in silica gels with the same microspectrophotometric apparatus and multiwavelengths analysis used in crystal experiments. The T state of hemoglobin in silica gels binds oxygen noncooperatively with an affinity and a Bohr effect similar to those observed in solution for the binding of the first oxygen molecule. Other allosteric effectors such as inositol hexaphosphate, bezafibrate, and chloride significantly affect oxygen affinity. Therefore, T state hemoglobins that are characterized by strikingly different functional properties share the absence of cooperativity in the binding of oxygen. These findings are fully consistent with the Monod, Wyman, and Changeux model and with most features of Perutz's stereocchemical model, but they are not consistent with models of both Koshland and Ackers.

The oxygen affinity of hemoglobin crystals is similar to that measured in solution in the presence of strong allosteric effectors such as inositol hexaphosphate, bezafibrate, and chloride (20–22). The oxygen affinity of hemoglobin crystals is similar to that measured in solution in the presence of strong allosteric effectors (23, 24). The absence of the Bohr effect is in keeping with Perutz’s stereocchemical mechanism (3) and with the three dimensional structures of partially (18, 25–27) or fully liganded hemoglobin (28, 29) showing intact salt bridges. A possible criticism to these studies is that hemoglobin in the crystal might not bind oxygen cooperatively within the T state due to the lattice forces that prevent relevant ligand-induced conformational changes. The encapsulation of deoxyhemoglobin in wet porous silica gels provides a way, which is an alternative to crystallization, of fixing the hemoglobin molecules in the T state (19). Silica gels have been shown to be chemically inert.
and to allow proteins to be incorporated retaining their specificity and reactivity (30–38). In particular, glass-encapsulated myoglobin and hemoglobin maintain their chemical and spectroscopic properties (31–33, 36) and have been used for the development of optical biosensors for the quantitative determination of oxygen, nitrogen monoxide, and carbon monoxide (31–33). Similar to hemoglobin crystals, wet silica gels contain about 50% water and 50% protein. Weak forces that contrast the quaternary transition are present and are probably associated with the interaction of silanol groups with hemoglobin (19). Moreover, protein-protein interactions are either absent because the size of the pores is such (<100 Å) (31) that only one hemoglobin tetramer is enclosed in a pore, or, if present, they are random. In the first report on deoxyhemoglobin encapsulated in silica gels by Shibayama and Saigo (19), oxygen was found to bind noncooperatively with an affinity about 2-fold higher than in the crystal. However, the effect of protons and other allosteric effectors was not investigated. In the present work, we have not only determined accurate oxygen-binding curves both in the absence and presence of the allosteric effectors inositol hexaphosphate and bezafibrate, but also the effect of protons and chloride on the oxygen affinity. Measurements were carried out with the same microspectrophotometric apparatus previously used in the determination of the oxygen binding by hemoglobin crystals (20–22, 39–44).

MATERIALS AND METHODS

Hemoglobin Purification—Human hemoglobin was purified as previously described (22).

Deoxyhemoglobin Encapsulation—The encapsulation of deoxyhemoglobin in silica gels was carried out following the procedure reported by Shibayama and Saigo (19) with some modifications. The silica sol was prepared using tetramethyl orthosilicate according to Ellerby et al. (30). A solution containing tetramethyl orthosilicate, water, and hydrochloric acid was thoroughly mixed in a glass vial and sonicated for 20 min at 4 °C. At this point of the procedure, practically no more tetramethyl orthosilicate is present since the half-time of the acid-catalyzed hydrolysis is about 2 min (45). An equal volume of a deoxygenated solution containing 10 mM phosphate, pH 6, was added to the sol and the resulting mixture was further deoxygenated for 40 min at 4 °C. A deoxygenated solution containing 3.6% w/v hemoglobin, 50 mM phosphate, 30 mM dithionite, pH 7.2, was added anaerobically to the sol. The mixture was distributed homogeneously on the wall of the vial. When the gel formed, a solution of 100 mM phosphate, 30 mM dithionite, pH 7, was layered on the gel. The silica wet gels were anaerobically stored at 4 °C.

Oxygen Binding Measurements—Before measuring, the silica gels were washed once with a fresh solution containing 100 mM phosphate, 30 mM dithionite, 1 mM EDTA, pH 7, four times with a solution containing 100 mM phosphate, 1 mM EDTA, and 6000 units/ml catalase, pH 8, and then four times with the same solution at the desired pH. The gels were loaded into a Dvorak-Stotler flow cell (46), covered by a gas permeable silicon copolymer membrane. The flow cell was mounted on the thermostatted stage of a Zeiss MPM03 spectrophotometer (47). The silica gels are optically isotropic and transparent (30, 31). Unpolarized light was used to record the light transmitted through the gel and the surrounding solution. Spectra were recorded in the wavelength range 480–670 nm at 1-nm intervals using gels that absorb usually less than 1 OD.

Oxygen pressures between 0 and 760 torr were prepared by mixing oxygen and helium with a gas mixture generator (Environics, series 200). The gas mixture was humidified by bubbling through a solution containing 100 mM phosphate buffer and passed through the flow cell and then to an oxygen meter. The oxygen electrode was calibrated with three certified oxygen gas mixtures, 1, 5, and 21% with pure oxygen.

The experiments were carried out by first exposing hemoglobin gels either to oxygen or to helium and then to a defined oxygen pressure. Spectra were usually recorded for at least 7 h to monitor both the equilibration with oxygen that is usually completed within 60 min and a slow successive process.

Data Analysis—The fractional saturation with oxygen and the fractional concentration of oxidized hemes were determined by fitting the complete spectrum to a linear combination of deoxy, oxy, and oxidized hemoglobin spectra (reference spectra) (22), recorded in solution, plus a baseline and a slope to take into account the nonperfect optical quality of the gel surface.

RESULTS AND DISCUSSION

The absorption spectra of hemoglobin silica gels were recorded as a function of time after the gas, passing through the flow cell where the gel is placed, changed from pure helium to an oxygen pressure of 160 torr (Fig. 1a). To determine the...
fractional saturation with oxygen and the fractional concentration of oxidized hemes by using all recorded wavelengths, the spectra were fitted to a linear combination of deoxyhemoglobin, oxyhemoglobin, and oxidized hemoglobin spectra (reference spectra) plus a baseline and a slope. A representative set of calculated and observed spectra is shown in Fig. 1, b-d. The quality of the fit is such that the two curves are exactly coincident. The change of the fractional saturation with oxygen as a function of time shows a complex kinetics characterized by a fast phase followed by a much slower one (Fig. 1e). The first phase is associated to the equilibration of the system (humidifier, gas lines, flow cell, and hemoglobin within the silica gel) to the new oxygen pressure. The second slow phase leads to a continuous increase of the fractional saturation and is most probably due to the T to R quaternary transition taking place within the silica gel (19). This interpretation is supported by the evidence that the slow phase is absent at fractional saturations lower than about 0.2 (Fig. 2). To obtain the fractional saturation of the T state hemoglobin at a defined oxygen pressure without the small contribution arising from the T to R transition, the slow phase was back extrapolated to time 0 assuming a linear dependence (Fig. 1e). The extrapolated value of the fractional saturation with oxygen does not appreciably depend on whether the gel was first equilibrated with helium or oxygen (Fig. 1e), thus demonstrating the reversibility of the process. This experiment was repeated for nine other oxygen pressures both in the absence and presence of the allosteric effectors inositol hexaphosphate and bezafibrate (Fig. 2). The binding of oxygen was analyzed by fitting the data to the equation $Y = p^n / (p^n + p_{50}^n)$ where $p$ is the oxygen pressure, $p_{50}$ is the oxygen pressure at half saturation and $n$ is the Hill coefficient (Fig. 3). The $p_{50}$ is 33.6 ± 0.5 torr and 74.2 ± 1.0 torr.

**Fig. 2.** Dependence of the fractional saturation with oxygen of hemoglobin and the fractional concentration of methemoglobin as a function of time at different oxygen pressures in the absence and presence of allosteric effectors. Fractional saturation with oxygen (●) and fractional concentration of oxidized hemes (■) are reported as a function of time for hemoglobin gels that were first equilibrated with helium and then exposed to the oxygen pressure indicated in each panel in the absence (panel a) and in the presence (panel b) of 2 mM inositol hexaphosphate and 2 mM bezafibrate.
in the absence and presence of allosteric effectors, respectively. The corresponding Hill plots are shown in Fig. 3, inset. A lower oxygen affinity was found by Shibayama and Saigo (19) possibly due to the limited set of data available and the analysis being carried out at a single wavelength. In solution, under close experimental conditions (100 mM phosphate buffer, pH 7.4, 25 °C), the value of $K_t$, the dissociation constant of the first oxygen that binds to the tetramer, was reported to be 74 torr and 199 torr in the absence and presence of inositol hexaphosphate, respectively (2). These values, corrected for temperature and pH differences (2, 12) give a $K_t$ of about 28 torr and 77 torr, indicating that interactions of silanol groups of gels with hemoglobin do not interfere with the modulation of oxygen affinity. The absence of any effect caused by the silica matrix on the structural and functional properties of hemoglobin molecules also holds for the R state of hemoglobin (19) and for a variety of other proteins including several enzymes (30–38).

The T state of hemoglobin in silica gels binds oxygen with a Hill coefficient of 0.80 ± 0.01 and 0.85 ± 0.01 in the absence and presence of allosteric effectors, respectively. A value of 0.86 was found by Shibayama and Saigo (19). A Hill coefficient lower than 1 indicates either a negative cooperativity or a site heterogeneity. The α-subunits exhibit a 2–5-fold higher affinity than β-subunits on the basis of kinetic (48), nuclear magnetic resonance (49), electron paramagnetic resonance studies (50), and oxygen binding studies in solution (51, 52) and in the crystalline state (20–22, 41). Different oxygen affinities for α- and β-hemes have been structurally explained by the presence in the heme pocket of the β-subunit of Val E11 that obstructs the access of the ligand to the iron (3, 4). In the absence of cooperativity, a value of 0.89 is obtained when a 4-fold difference in oxygen affinity between α- and β-heme is assumed (22).

An analysis of the data reported in Fig. 3, which assumes no cooperativity and two classes of heme sites equally populated, gives an affinity of the α-heme 8.7- and 6-fold greater than the β-heme in the absence and presence of allosteric effectors, respectively. The calculated α-β inequivalencies are much greater than those observed either in solution or in the crystalline state, suggesting that this analysis is oversimplified. In fact, the T state of hemoglobin might possess another source of heterogeneity that should be taken into account. On the basis of the model proposed by Rivetti et al. (22), deoxyhemoglobin in solution exists as a distribution of molecules with intact and broken salt bridges that possess low and high oxygen affinities, respectively. An analysis of the oxygen binding curves of hemoglobin in silica gels, with and without allosteric effectors, that assumes a 2–5-fold α-β inequivalence and two distinct populations of hemoglobin molecules with either intact or broken salt bridges does not lead to reliable values due to the limited set of available data. However, when the data were fitted, assuming that (i) the α-subunits with broken salt bridges exhibit a $P_{50}$ of 4.1 torr, the value determined for $\alpha$(Fe$^{2+}$)$_2$βNi$^{2+}$ hybrid at pH 8.5 and corrected for temperature difference (51); (ii) the α-subunits with intact salt bridges exhibit a $P_{50}$ of 94 torr, the value determined in hemoglobin crystals (22); and (iii) the α-β inequivalence is 2–5-fold, the fraction of sites with broken salt bridges was actually found to decrease from about 50% to 25% in the absence and presence of allosteric effectors, respectively.

These results indicate that the T state of hemoglobin in silica gels binds oxygen without any degree of cooperativity, and in conjunction with the results obtained on hemoglobin crystals, limit the validity of most of the models so far proposed to explain the hemoglobin function. Furthermore, they provide evidence that any heme-heme interaction in the T state detected either spectroscopically or crystallographically (4, 25, 26, 29) does not affect the oxygen affinity of the other subunits.

In solution, an important feature associated with the binding of oxygen by the T state of hemoglobin is the release of protons, the so called tertiary Bohr effect (3, 4, 12). This behavior has been explained by the oxygen-induced conformational changes that lead to the breakage of salt bridges (3, 4). Therefore, a test of the functional integrity of the T state hemoglobin in silica gels is the determination of the effect of protons on the oxygen affinity. We measured the dependence on pH of the fractional saturation with oxygen of hemoglobin silica gels exposed to an oxygen pressure of 40 torr both in the absence and presence of allosteric effectors (Fig. 4a). It was not possible to obtain reproducible data over pH 8 because hemoglobin molecules escape from the silica gel. This behavior might be caused either by an increase of pore size as a function of pH and/or by a pH-dependent dimer-tetramer dissociation with dimers escaping the gel (53). Assuming that the Hill coefficient of the T state hemoglobin is pH-independent, the corresponding $P_{50}$ was calculated (Fig. 4b). Oxygen affinity increases about 2-fold between pH 6.3 and 7.5 in the absence of chloride, and 2.7-fold between pH 6.3 and 8.0 in the presence of 200 mM chloride, closely paralleling the effects on $K_t$ found in solution (2, 12). In the presence of inositol hexaphosphate and bezafibrate, the increase of $P_{50}$ over the same pH range is 3.8-fold. In solution, the effect of pH on $K_t$ in the presence of inositol hexaphosphate is only slightly greater than in its absence, whereas the overall Bohr effect is significantly greater (2). Furthermore, in hemoglobin silica gels the Bohr coefficient, $\Delta H^+ = d\log P_{50}/d\log [pH]$, is $-0.19$ at pH 7.4 in the absence of chloride, $-0.35$ at pH 7.4 in 200 mM chloride, and $-0.55$ at pH 7.9 in the presence of 2 mM inositol hexaphosphate and 2 mM bezafibrate. In solution, a $\Delta H^+$ of $-0.28$ and $-0.50$ was determined from the dependence of $K_t$ in 5 mM and 150 mM chloride (12, 54). Very similar values were determined for the overall Bohr effect (4,
In the presence of 2,3-diphosphoglycerate or inositol hexaphosphate the value was \(2(0.7–0.8)\) (2, 4). The comparison of these values indicates that the tertiary Bohr effect accounts for most of the protons released upon oxygen binding, which is in agreement with Perutz’s stereochemical mechanism (3, 4).

The difference in the number of Bohr protons released on oxygenation under different experimental conditions indicates that the change of pKs of ionizable residues from the deoxy to the oxy state within a T state is controlled by the binding of other allosteric effectors, as also observed for the overall Bohr effect (55). Moreover, as noted in solution, the maximal Bohr coefficient of the T state hemoglobin is observed at about pH 6.5 and 7.4 in the absence and presence of chloride, respectively, and at a pH between 7.5 and 8.0 in the presence of inositol hexaphosphate and bezafibrate, indicating a progressive shift of pK of the ionizable residues that are linked to oxygenation.

The effect of chloride on oxygen affinity has recently been explained by a new kind of allosteric regulation (56). Chloride decreases oxygen affinity by neutralizing the excess positive charge present in the central cavity of the T state hemoglobin and, consequently, stabilizing the T state. We found that chloride decreases the oxygen affinity of the T state hemoglobin in silica gels by 2-fold (Fig. 5), which is close to the 3-fold effect on \(K_1\) in solution (2). According to the Rivetti et al. (22) model, this finding indicates an increase of the population of unliganded T state hemoglobin molecules with intact salt bridges. Indeed, a higher Bohr effect is observed in the presence of chloride than in its absence.

CONCLUSIONS

The T state of hemoglobin in silica gels is modulated by protons and other allosteric effectors very much in the same way as in solution for the binding of the first oxygen. Therefore, no conformational restriction seems to be imposed by the silica matrix on tertiary conformational changes associated with oxygen binding and effectors regulation. The observed properties unequivocally demonstrate that the T state of hemoglobin binds oxygen noncooperatively in full agreement with that found in crystals of human hemoglobin in the absence and presence of allosteric effectors (20–22) despite dramatic differences in the oxygen affinity, the Bohr effect, and the influence of other allosteric effectors. The lack of cooperativity of T state hemoglobin is correctly predicted by the Monod, Wyman, and Changeux model (5–7) and cannot be reconciled with Ackers’ model (14, 15), which indicates a 36-fold increase of the oxygen affinity within the \(\alpha_1\beta_1\) dimer in the T state, as well as with any model that postulates ligand-induced changes of oxygen affinity within the T state (3, 10, 13).

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