Subunit heterogeneity in the structure and dynamics of hemoglobin

A transient Raman study

Thomas W. Scott, Joel M. Friedman†, Masao Ikeda-Saito+ and Takashi Yonetani+

Bell Laboratories, Murray Hill, NJ 07974 and +*Department of Biochemistry and Biophysics, University of Pennsylvania School of Medicine, Philadelphia, PA 19104, USA*

Received 17 May 1983

Abstract and keywords not received

1. INTRODUCTION

Hemoglobin (Hb) is a tetrameric protein consisting of two α- and two β-subunits. Each of these subunits contains a heme group to which oxygen can reversibly bind. Although the oxygen binding sites are very similar in the α- and β-subunits, both X-ray crystallographic studies [1] and spectroscopic studies [2–6] indicate subunit-specific differences in the heme pocket. However, it has yet to be fully explored to what extent these various structural differences are functionally relevant. Results of ligand binding studies on hemoglobin [7–10] do suggest varying degrees of subunit heterogeneity with respect to ligand reactivity. Recent results [11–14] from transient Raman studies on hemoglobin indicate a direct relationship between the iron–proximal histidine linkage in Hb*(t), the transient photoproduct occurring at a time t subsequent to the photodissociation of a ligated hemoglobin, and the barrier height regulating both geminate recombination [15–17] and possibly the microscopic off rates. Therefore, it is of interest to examine the subunit specificity of the time evolution in the iron–proximal histidine linkage over a time scale that is appropriate for evaluating its effect upon geminate recombination and spontaneous dissociation. Here, we describe results obtained using time-resolved Raman scattering to follow the subunit-specific evolution of the iron–proximal histidine linkage in transient species derived from photodissociated carboxy iron–cobalt hybrid hemoglobins.

† To whom correspondence should be addressed

2. MATERIALS AND METHODS

The Fe–Co hybrid hemoglobins were prepared as in [2]. The carboxy derivatives were prepared by first adding a few grains of dithionite to an oxygen free solution of hybrid hemoglobin and then saturating the solution with high purity CO. Only the iron porphyrins bind the CO, consequently the oxygen free solution contains hybrids that are half saturated with CO. During the experiment, solutions ($\sim 10^{-4}$ M in heme) were maintained at 35°C to minimize the yield of geminate recombination which would complicate the measurements of the structural relaxation [18].

An initial 10 ns laser pulse (1 mJ) at 4050 Å (Molection) was used to completely photolyze the sample. An electronically delayed second 10 ns pulse at 4350 Å (~1 mJ) from an excimer laser pumped dye laser (Lambda Physics 2002) generated the Raman spectrum of the photolyzed material. A detailed account of the experimental apparatus is described in [14]. Using this excitation frequency there are no detectable contributions in the spectra from the cobalt porphyrins.

Published by Elsevier Science Publishers B.V.

3. RESULTS AND DISCUSSION

In fig.1 is shown the time evolution of the Raman frequency of the Fe–His stretching mode of the transient deoxy species derived from the photodissociation of both α(carboxyFe)$_2\beta$(Co)$_2$ and α(Co)$_2\beta$(carboxyFe)$_2$ at pH 9 and pH 7.0. Representative spectra of the photoproduct at 10 ns showing the Raman band associated with Fe–His stretching mode are shown in fig.2. Although the samples used for fig.2 were at 5°C, there were no detectable temperature effects on the frequencies of this Raman band in the 10 ns spectra.

Fig.1 indicates that at pH 9 the initial frequencies and subsequent relaxation of Fe–His mode in both transient hybrids are the same. It is also seen that at pH 7.0 in the presence of IHP (3 mM) there is a large difference between the two hybrids. The frequencies from the pH 7 transient derived from α(carboxyFe)$_2\beta$(Co)$_2$ are, for all the delays, substantially lower than from the other hybrid. The frequencies at 10 ns from both pH 9 transients and from the α(Co)$_2\beta$(carboxyFe)$_2$ transient at pH

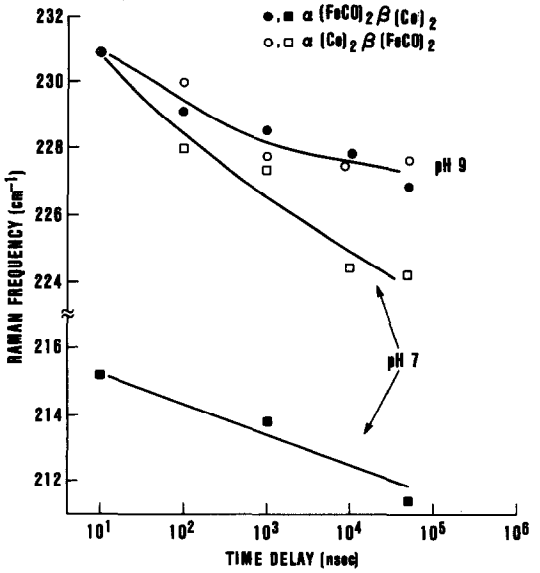

Fig.1. The time evolution of the iron–proximal histidine stretching mode in the deoxy photoproduct generated by the photolysis of carboxy-Fe–deoxy-Co hybrid hemoglobins. The times refer to the delay subsequent to the 10 ns long photolysis pulse. The pH 7 solutions also contained IHP (~3 mM).

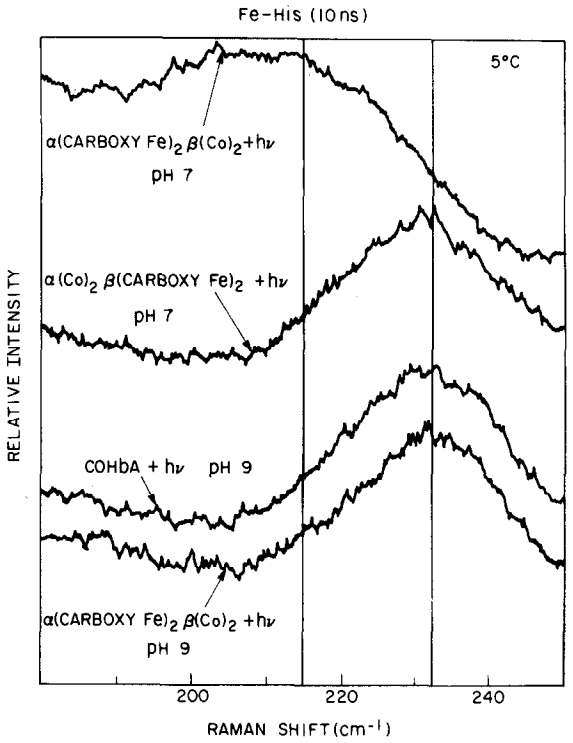

Fig.2. The Raman band associated with the iron–proximal histidine stretching mode in several transient hybrid hemoglobins occurring within 10 ns of photolysis. The solutions at pH 7 also contained IHP (~3 mM).

7.0 are all equivalent having a value of ~230 cm^{-1}. This is the same frequency observed for photolyzed COHbA at 10 ns over the same pH range [14]. The time evolution [14,19] of this frequency in HbA at pH 9 and and pH 7 also resembles very closely the top two relaxation curves respectively shown in fig.1. The frequencies on the bottom curve are all considerably lower than any of the values observed [14,19] between 10 ns and 10 μs for HbA over pH 9.0–6.2 (+ IHP).

Raman studies on steady states [6,20] and transient forms [11,13,21–23] of deoxy hemoglobin indicate that the frequency of the Fe–His stretching mode is sensitive to quaternary structure. For equilibrium species, this frequency increases from ~215–222 cm^{-1} on going from the *T–R* quaternary structure. Time-resolved Raman studies [10,13,23] have also shown that in addition to being sensitive to quaternary structure, the frequency of the Fe–His stretching frequency reflects the re-

cent history (<10 μs) of the deoxy heme pocket with respect to ligation. In comparing a deoxy Hb to the corresponding deoxy Hb(t)*, the photoproduct at time t, with both having the same quaternary structure, the frequency of the Fe–His stretching mode is invariably higher for the transient when t, the delay subsequent to photolysis, is short compared to the protein relaxation time. For example on going from T state deoxy Hb to T state deoxy Hb* (10 ns) [e.g., photolyzed NOHbA or COHb (Kansas) at pH 6.5 + IHP] the frequency increases from ~215–222 cm^{-1}. Similarly, this frequency increases from ~222–230 cm^{-1} in going from R state deoxy Hb [e.g., Hb(Kempsey) at pH 8.5] to deoxy Hb* (10 ns) [e.g., photolyzed COHbA or COHb (Kempsey) at pH 7 or higher]. Thus, the protein changes induced by both ligation and the T–R quaternary state transitions result in a protein configuration about the heme that is associated with an increase in the frequency of the Fe–His stretching mode. Similar behavior is reflected in the frequency of the oxidation state marker band at ~1355 cm^{-1} [12]. This frequency varies in an inverse linear fashion with that of the Fe–His mode for both equilibrium [20] and transient species [23]. Since the frequency of this marker band is sensitive to the Π electron distribution in the porphyrin macrocycle, the above correlation supports the idea that variations in the iron–histidine linkage can modulate the Π electron density in the heme [24].

The sensitivity of the Fe–His mode to ligation and quaternary structure induced changes allows us to use the frequencies in fig.1 to unambiguously establish the quaternary state of the initial ligated hybrids. It is observed that the frequency of the Fe–His mode is 230 cm^{-1} for the transients at 10 ns derived from both α(carboxyFe)$_2\beta$(Co)$_2$ and α(Co)$_2\beta$(carboxyFe)$_2$ at pH 9 and α(Co)$_2\beta$(carboxyFe)$_2$ at pH 7 + IHP. This frequency is characteristic of deoxy Hb* (10 ns) derived from ligated high affinity R state hemoglobins. Thus, there is no doubt that the above half-ligated hybrid hemoglobins are also R state species. On the other hand, the low frequency of ~215 cm^{-1} seen for the 10 ns transient derived from α(carboxyFe)$_2\beta$(Co)$_2$ at pH 7.0 + IHP is well within the range for a T state hemoglobin. These results demonstrate that whereas at high pH, the static heme environment for an R state ligated subunit is not detectably influenced by ligation in the other subunits, at low pH the heme environment about the ligated α-subunits is highly dependent upon the degree of saturation in the β-subunits. Consistent with this observation is the idea that heme–heme interactions are mediated through the α_1–β_2 interface. At high pH, the stability of this interface for partially or completely saturated Hb is primarily determined by the heterotropic factor whereas at low pH ligation plays a much greater role in stabilizing the interfacial contacts. Our results show that upon decreasing the external forces that stabilize the R structure by a lowering of the pH [25] and or adding IHP, the contribution to the R state stability from ligand binding in the α-subunits is significantly less compared to ligand binding in the β-subunits. A similar conclusion was reached using paramagnetic resonance techniques [2,26]. This finding raises the interesting question as to whether, under conditions favoring decreased affinity such as low pH, there might be a subunit specific variation in the ligand off rates which would favor deligation of the β-subunits over α-subunits. At pH 7, a partially ligated hemoglobin with deoxy β-subunits would be more likely to undergo the $R \longrightarrow T$ quaternary switch. Indeed there are studies indicating that the β subunit is more likely to remain ligand free subsequent to dissociation. It is observed [12] that under conditions favoring the T structure, sub-ns geminate recombination favors religation of the α-subunit over the β-subunit. Similarly, measurements of off rates also indicate that for oxyHb it is the β-subunit which tends to more readily lose the ligand via spontaneous dissociation [7,8]. Those partially ligated intermediates with ligand free β-subunits would then be most likely to undergo the $R \longrightarrow T$ switch which results in an increase in the off rates for the remaining ligated sites. A manifestation of this increased off rate in the T state species is in evidence in the top spectrum of fig.2 where it can be seen that the broad Raman band associated with the T state transient (α(carboxyFe)$_2\beta$(Co)$_2$) has considerable intensity extending towards 200 cm^{-1}. Studies [6] on the steady deoxy hybrids have revealed that the average frequency of this mode for the deoxy α-subunit is between 200 and 210 cm^{-1}. The line shape and position of the Raman band of the 10 ns transient is consistent with starting material that contains both ligated

and spontaneously dissociated α-subunits. In contrast the spectrum of other hybrids, all in the *R* state, show no evidence of a population of spontaneously dissociated carboxy hemes.

Aside from being useful in characterizing the quaternary structure of both stable and transient forms of Hb, the Raman spectrum contains information directly linking structure with function. In a recent analysis [13,14] of the Fe–His mode, it was suggested that the variation in this Raman frequency originates from changes in the tilt of the proximal histidine with respect to the heme plane. Increasing the tilt increases the repulsive forces between the imidazole and the pyrrol nitrogens which reduces the frequency of the Fe–His stretching mode. Furthermore, the increased repulsive force makes it energetically more difficult to move the iron in plane upon ligand binding [23]. Consequently frequency changes of Fe–His mode should be correlated with variation in both the heme-associated potential energy barrier regulating ligand binding and the stability of the ligated heme. Such a relationship does appear to exist. It has been noted [14] that for the 10 ns transients of a given hemoglobin, an induced (pH, phosphate, Cl^-) decrease in the frequency of the Fe–His mode (or an increase in the oxidation state marker band [12]) is associated with a decrease in geminate recombination and an increase in the rate of spontaneous dissociation.

In fig.1 we see that on going from pH 9–7 the *R* state species all have the same initial frequency at 10 ns. Subsequent relaxation of the deligated heme pocket represents a shift in the equilibrium from the *R* state ligated heme pocket configuration (230 cm^{-1}) to the *R* state deoxy configuration (222 cm^{-1}) [14]. From fig.1 it can be seen that this relaxation is faster for the above pH 7 sample. Since this pH-related difference in the Fe–His relaxation is evident even over the ~200 ns associated with geminate recombination, there should be fewer recombined sites for the low pH sample. Because the dissociation-induced shift in the *R* state equilibrium from the ligated to deoxy heme pocket configuration is sustained by increasing the interval over which the heme remains ligand free, the effect of lowering the pH is to create a shift in the averaged heme environment by a dynamic decrease in the rebinding subsequent to ligand dissociation. Thus observed [27,28] pH-induced differences in both ligand off rates and affinities in *R* state hemoglobin may originate not from static structural differences but from pH-related differences in the kinetic constants associated with the ligated-deoxy tertiary structure equilibrium within the *R* state.

REFERENCES

[1] Baldwin, J.M. and Chothia, C. (1979) J. Mol. Biol. 129, 175–220.
[2] Ikeda-Saito, M., Yamamoto, H. and Yonetani, T. (1977) J. Biol. Chem. 252, 8639–8644.
[3] Ogawa, S. and Shulman, R.G. (1972) J. Mol. Biol. 70, 315–336.
[4] Takahashi, S., Lin, A.K.L.C. and Ho, C. (1980) Biochemistry 19, 5196–5202.
[5] Sugita, Y. (1975) J. Biol. Chem. 250, 1251–1256.
[6] Ondrias, M.R., Rousseau, D.L., Kitagawa, T., Ikeda-Saito, M., Inubushi, T. and Yonetani, T. (1982) J. Biol. Chem. 257, 8766–8770.
[7] Gray, R.D. and Gibson, Q.H. (1977) J. Biol. Chem. 252, 7538–7547.
[8] Olson, J.S., Anderson, M.E. and Gibson, Q.H. (1971) J. Biol. Chem. 246, 5919–5923.
[9] Viggiano, G. and Ho, C. (1979) Proc. Natl. Acad. Sci. USA 76, 3673–3677.
[10] Ikeda-Saito, M. and Yonetani, T. (1980) J. Mol. Biol. 138, 845–858.
[11] Friedman, J.M., Stepnoski, R.A. and Noble, R.W. (1982) FEBS Lett. 146, 278–282.
[12] Friedman, J.M., Stepnoski, R.A., Stavola, M., Ondrias, M.R. and Cone, R.L. (1982) Biochemistry 21, 2022–2028.
[13] Friedman, J.M., Rousseau, D.L., Ondrias, M.R. and Stepnoski, R.A. (1982) Science 218, 1244–1246.
[14] Friedman, J.M., Scott, T.W., Ikeda-Saito, M. and Yonetani, T. (1983) J. Biol. Chem., in press.
[15] Duddell, D.A., Morris, R.J. and Richards, J.T. (1979) J.C.S. Chem. Comm. 75–78.
[16] Alpert, B., El Mohsni, S., Lindqvist, L. and Tfibel, T. (1978) Chem. Phys. Lett. 64, 11–15.
[17] Friedman, J.M. and Lyons, K.B. (1980) Nature 284, 570–573.
[18] Lyons, K.B. and Friedman, J.M. (1982) in: Hemoglobin and Oxygen Binding (Ho, C. ed) pp.333–337, Elsevier, Amsterdam, New York.
[19] Scott, T.W. and Friedman, J.M. (1983) submitted.
[20] Ondrias, M.R., Rousseau, D.L., Shelnutt, J.A. and Simon, S.R. (1982) Biochemistry 21, 3428–3437.
[21] Irwin, M.J. and Atkinson, G.H. (1981) Nature 293, 317–318.

[22] Stein, P., Turner, J. and Spiro, T.G. (1982) J. Phys. Chem. 86, 168–170.
[23] Friedman, J.M. (1983) in: Time Resolved Vibrational Spectroscopy (Atkinson, G. ed) Academic Press, London, New York, in press.
[24] Perutz, M.F. (1980) Proc. R. Soc. London B 208, 135–162.
[25] Chu, A.H. and Ackers, G.K. (1981) J. Biol. Chem. 256, 1199–1205.
[26] Ikeda-Saito, M., Inubushi, T., McDonald, G.G. and Yonetani, T. (1978) J. Biol. Chem. 253, 7134–7137.
[27] McDonald, M.J. and Noble, R.W. (1972) J. Biol. Chem. 247, 4282–4287.
[28] DeYoung, A., Pennelly, R.R., Tan-Wilson, A.L. and Noble, R.W. (1976) J. Biol. Chem. 251, 6692–6698.