

## Ligand Kinetics in Hemoglobin Hiroshima

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**ABSTRACT** Hemoglobin Hiroshima is an electrophoretically fast-moving variant with a fourfold increase in oxygen affinity and a decreased Bohr effect. Based on a decreased rate of dissociation of O<sub>2</sub> in the presence of dithionite and an increased rate of binding of CO by the deoxy form, we have concluded that the kinetic basis of the high affinity exhibited by Hb Hiroshima is the concurrence of a faster combination rate and a slower dissociation rate for ligands.

### INTRODUCTION

Hemoglobin Hiroshima, discovered by Hamilton, Iuchi, Miyaki, and Shibata (1) in several members of a Japanese family, is a fast-moving beta chain variant associated with increased oxygen affinity and compensatory erythrocytosis. The present communication deals with a preliminary account of the kinetic basis of the oxygen equilibria of this hemoglobin variant.

### METHODS

Venous blood from a donor known to be heterozygous for Hb Hiroshima was sent from Japan to New York in iced containers and processed within 48 hr.

Hemolysates were prepared by the method of Drabkin (2) with minor modifications. Hemoglobin Hiroshima was isolated by starch block electrophoresis (3) using veronal buffer, 0.04 mole/liter at pH 8.6, and the variant, which

migrated cathodally to Hb A, was eluted from the starch with 0.001 M phosphate buffer (pH 7). The purity of the isolated hemoglobin was verified by starch gel electrophoresis (Tris-borate buffer pH 8.6) (4). Haptoglobin comprised about 90% of a preparation which was purified according to the previously described method (5).

The binding of CO by hemoglobin and the reaction of oxyhemoglobin with dithionite were measured by the stopped flow method using the apparatus of Gibson and Milnes (6) with some modifications. An analogue-to-digital converter model 130 E; (Digital Equipment Corp., Maynard, Mass.) was used to digitize the photomultiplier output which was then transferred to a DECPDP 8/I computer under control of a program described by DeSa and Gibson (7).

Hemoglobin binding to haptoglobin was measured by the method of Nagel and Gibson (5) based on the quenching of the aromatic amino acid fluorescence by the hemes of hemoglobin. Excitation at 287 mμ was used, and light was obtained from a 150 w direct current xenon lamp and a Bausch and Lomb 250 mm grating monochromator. An EMI photomultiplier type 9525-B and a Corning glass filter No. 7-60 detected the protein fluorescence at 350 mμ. As in the absorbance stopped flow experiments, the data were collected directly into the memory of a PDP-8/I computer, and simultaneous visual observations of the reaction was possible with an oscilloscope.

### RESULTS

*Binding of CO.* The kinetics of the reaction between deoxy-Hb A and deoxy-Hb Hiroshima with carbon monoxide is shown in Fig. 1. Hemoglobin solutions were 24 μmoles/liter (heme) before mixing, and in 0.1 M potassium phosphate buffer, pH 7.0. The carbon monoxide solutions were prepared by bubbling buffer with pure CO at 1 atm of pressure and diluted with oxygen-free 0.1 M potassium phosphate buffer, pH 7.0. The final concentration of carbon monoxide after mixing was 97.5

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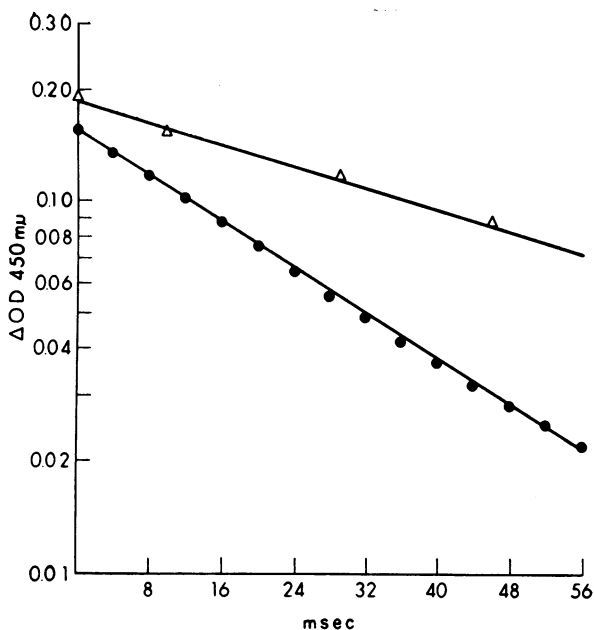


FIGURE 1 Kinetics of the combination reaction between carbon monoxide and Hb A and Hb Hiroshima, determined by stopped flow method;  $\triangle$  ---  $\triangle$ , Hb A;  $\bullet$  ---  $\bullet$ , Hb Hiroshima. Abscissa: milliseconds. Ordinate: change in optical density at 450  $m\mu$ . Temperature: 22°C; 0.1 M potassium phosphate buffer, pH 7.0. Hemoglobin concentration: 24  $\mu$ moles/liter (heme) before mixing. CO concentration: 97.5  $\mu$ moles/liter (after mixing).

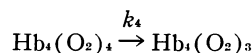
$\mu$ moles/liter. Dithionite was added to the deoxy-Hb solution and the CO-phosphate buffer, which were subsequently mixed in equal volumes in the stopped flow apparatus. The reaction was followed at 450  $m\mu$ , and the experiments were performed at 22°C. The second-order constant for Hb A is  $1.8 \times 10^5 \text{ M}^{-1} \cdot \text{sec}^{-1}$  and  $3.6 \times 10^5 \text{ M}^{-1} \cdot \text{sec}^{-1}$  for Hb Hiroshima.

*The reaction of oxy-Hb and dithionite.* The solutions of Hb A and Hb Hiroshima were 24  $\mu$ moles/liter (before mixing) and in 0.1 M potassium phosphate buffer, pH 7.0. These solutions were mixed with a 0.2% dithionite solution in a stopped flow apparatus. The reaction was followed at 541.5  $m\mu$  and at 22°C, and the results are shown in Fig. 2. The  $k$  obtained for Hb A was 35  $\text{sec}^{-1}$ , and for hemoglobin Hiroshima it was 20  $\text{sec}^{-1}$ .

*The replacement of oxygen by carbon monoxide.* The displacement reaction of  $\text{O}_2$  from oxy-Hb by CO was studied in the stopped flow apparatus, and the results are shown in Fig. 3. One syringe contained Hb Hiroshima diluted with a 0.1 M potassium buffer, pH 7.0, equilibrated with different concentrations of  $\text{O}_2$ . The other syringe contained the same buffer saturated with CO. The reciprocal of the observed rate of replacement of oxygen by carbon monoxide, expressed as a rate constant vs. the  $\text{O}_2$  concentration, is depicted in Fig. 3.

The intercept in this case is  $4/k_4$ , as demonstrated by Gibson and Roughton (8).

The dissociation velocity constant,  $k_4$ , corresponds to



In the case of Hb Hiroshima,  $k_4$  is 33  $\text{sec}^{-1}$  as compared with 55  $\text{sec}^{-1}$  for Hb A under similar conditions.

*The binding of hemoglobin by haptoglobin.* This reaction was studied with a stopped flow apparatus provided with a fluorescence attachment. The reaction was followed by utilizing the fluorescence quenching of the 350  $m\mu$  emission of haptoglobin by the hemes of hemoglobin. Solutions of Hb A and Hb Hiroshima, 25  $\mu$ moles/liter (heme), in 0.1 M potassium phosphate buffer, pH 7.0 were reacted with a 3.05  $\mu\text{M}$  solution of haptoglobin at 22°C; the results are shown in Fig. 4. The pseudo first-order combination rate is 1.1  $\text{sec}^{-1}$  for Hb A and 1.9  $\text{sec}^{-1}$  for Hb Hiroshima.

## DISCUSSION

Hemoglobin Hiroshima was initially described as the substitution of  $\beta$  143-histidine by aspartic acid. Perutz et al. (personal communication) has recently completed an X-ray study of deoxy-hemoglobin Hiroshima, which demonstrated that the difference electron density maps at 3.5 Å resolution are featureless in the region of His 143 but show a strong positive peak representing aspartate at position 146. The final definition of the site of the substitution will require further chemical studies.

Hemoglobin Hiroshima has been shown to exhibit an increased affinity for oxygen (1, 9). At pH 7.0, the purified solution of Hb Hiroshima has a  $P_{50}$  (pressure

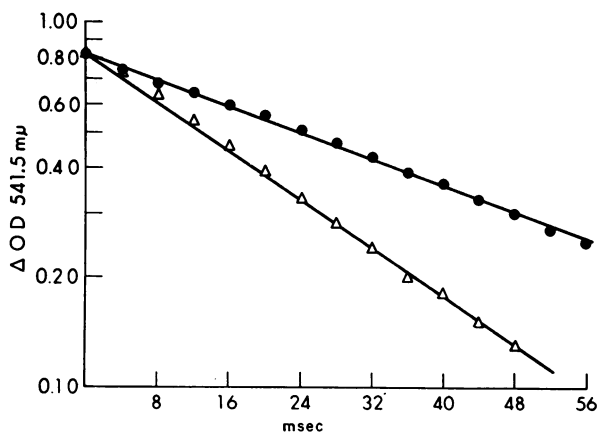


FIGURE 2 Kinetics of the reaction of oxy Hb A and Hb Hiroshima with dithionite. Abscissa: milliseconds. Ordinate: change in optical density at 541.5  $m\mu$ . Temperature: 22°C; 0.1 M potassium phosphate buffer; pH 7.0. Hemoglobin concentration: 24  $\mu$ moles/liter (heme) before mixing.  $\triangle$  ---  $\triangle$ , Hb A;  $\bullet$  ---  $\bullet$ , Hb Hiroshima.

of O<sub>2</sub> at 50% saturation), which is 4 times higher than that of a solution of Hb A. The Bohr effect (the variation of oxygen affinity with pH) is considerably reduced in this variant: the  $\Delta \log P_{50}/\Delta \text{pH}$  ratio is reduced by half in the range between pH 7.4 and 7.0 (1).

Another interesting feature of the oxygen equilibria of Hb Hiroshima is the presence of moderately decreased heme-heme interaction. The Hill equation (10) describes empirically the oxygen equilibria of Hb,

$$\log (s/1-s) = n \log p + \log k,$$

where  $s$  represents the fractional saturation with oxygen,  $p$  is the partial pressure of oxygen, and  $n$  is a constant representing in a general way the stabilizing interactions between the oxygen-binding sites. An  $n$  value of 1 represents no heme-heme interaction, and  $n = 4$  represents the maximal possible interaction in a tetrameric molecule that contains four binding sites.

In Hb Hiroshima, at pH 7.0,  $n = 2.0$ , rather than 2.7–3.0 as classically found in Hb A. Furthermore, the  $n$  value varies with pH and increases to 2.6 at pH 7.8 when continuous recording methods are employed. This is in contrast with the behavior of  $n$  in Hb A, which varies much less with pH. An intriguing feature of the oxygen equilibria of Hb Hiroshima has been observed: at low pH, the curve is biphasic (1, 9).

The data described here show that the increased ligand affinity is probably due to changes in both binding and dissociation reactions, though our measurements have so far involved different ligands for the two types of kinetic reactions which can most accurately and easily be studied using carbon monoxide for binding and oxygen for dissociation.

It has been recently demonstrated that most of the cooperativity between hemes in hemoglobin is due to changes in the dissociation constants (11, 12). Hence,

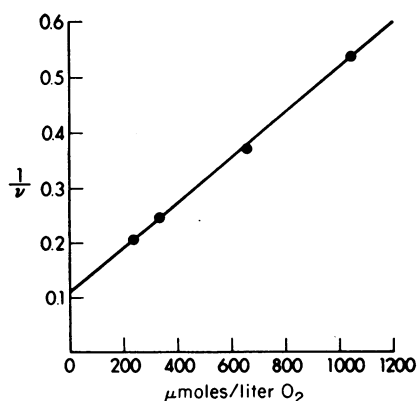


FIGURE 3 Kinetics of the replacement of O<sub>2</sub> bound to Hb Hiroshima by carbon monoxide at different O<sub>2</sub> concentrations. Plot of the reciprocal rate constant of the replacement reaction vs. O<sub>2</sub> concentration before mixing. Temperature: 22°C; 0.1 M potassium phosphate buffer, pH 7.0.

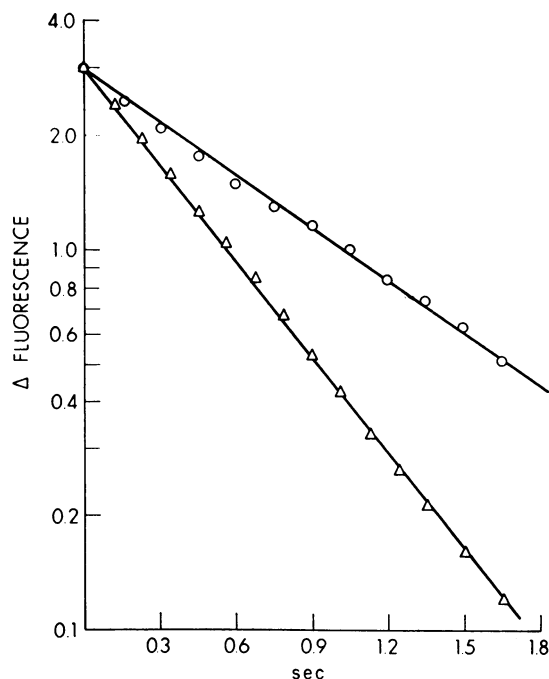


FIGURE 4 Combination rate of haptoglobin with Hb A and Hb Hiroshima, studied by fluorescence quenching. Haptoglobin solution, 3.05  $\mu\text{moles/liter}$ ;  $\bigcirc$  ---  $\bigcirc$ , Hb A, 25  $\mu\text{moles/liter}$  (heme);  $\triangle$  ---  $\triangle$ , Hb Hiroshima, 25  $\mu\text{moles/liter}$  (heme). All solutions in 0.1 M potassium phosphate, pH 7.0. Temperature: 22°C; concentrations before mixing.

a difference in rates should be observed between the dissociation velocity constant  $k_1$  of the first site in a fully liganded molecule and the dissociation velocity constant obtained with dithionite, which is greatly influenced by the dissociation of the rest of the ligands in the tetramer.

In Hiroshima the ratio between  $k_1/4$  (8.25  $\text{sec}^{-1}$ ) and the dissociation rate measured by dithionite (20  $\text{sec}^{-1}$ ) is only slightly lower than that of Hb A: 13  $\text{sec}^{-1}$  and 35  $\text{sec}^{-1}$ , respectively. This demonstrates the presence of apparent differences in the dissociation constants for each of the hemes, implying cooperativity between binding sites, a phenomenon which has classically been referred to as "heme-heme interaction."

The variation of  $n$  with pH and the biphasic character of the oxygen equilibrium curve at low pH, cannot be explained with the available data, and further work is in progress to resolve this problem.

The moderate decrease in heme-heme interaction cannot be fully explained with the present data. Nevertheless, a substitution in the C terminal portion of the H helix, which interferes with heme-heme interaction, is consistent with an important role for Tyr HC2(145) $\beta$  in the underlying mechanism of cooperativity. Early suggestions of the participation of this residue in the

heme-heme interaction mechanism (13) have been further developed and supported by the findings of Moffat (14) and Perutz (15).

Since the binding between hemoglobin and haptoglobin appears to occur exclusively via the hemoglobin dimer at micromolar levels of concentration (16), the slight increase in the rate of the binding observed with Hb Hiroshima may be due to a small increase in the dissociability of this hemoglobin variant or a modification of the binding site by the substitution.

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*Note added in proof:* Since the submission of this article for publication, the structure of Hb Hiroshima has been established. X-ray diffraction crystallography of this mutant, confirmed by independent chemical analysis, shows that the amino acid substitution aspartate for histidine is at position  $\beta$ -146 (HC3) and not 143(H21) as previously reported. (Perutz, del Pulsinelli, Eyck, Kilmartin, Shibata, Miyaji, Iudi, and Hamilton. 1971. *Nature (London)*. [In press]).

## REFERENCES

1. Hamilton, H. B., I. Iuchi, T. Miyaki, and S. Shibata. 1969. Hemoglobin Hiroshima ( $\beta^{148}$  histidine  $\rightarrow$  aspartic acid): a newly identified fast moving beta chain variant associated with increased oxygen affinity and compensatory erythremia. *J. Clin. Invest.* **48**: 525.
2. Drabkin, D. L. 1946. Spectrophotometric studies. XIV. The crystallographic and optical properties of the hemoglobin of man in comparison with those of other species. *J. Biol. Chem.* **164**: 703.
3. Kunkel, H. G., R. Cepellini, V. Muller-Eberhard, and J. Wolf. 1957. Observations on the minor basic hemoglobin component in the blood of normal individuals and patients with thalassemia. *J. Clin. Invest.* **36**: 1615.
4. Smithies, O. 1959. An improved procedure for starch gel electrophoresis. *Biochem. J.* **71**: 585.
5. Nagel, R. L., and Q. H. Gibson. 1967. Kinetics and mechanism of complex formation between hemoglobin and haptoglobin. *J. Biol. Chem.* **242**: 3428.
6. Gibson, Q. H., and L. Milnes. 1964. Apparatus for rapid and sensitive spectrophotometry. *Biochem. J.* **91**: 161.
7. DeSa, R. J., and Q. H. Gibson. 1969. A practical automatic data acquisition system for stopped-flow spectrophotometry. *Comput. Biomed. Res.* **2**: 494.
8. Gibson, Q. H., and F. J. W. Roughton. 1955. The kinetics of dissociation of the first oxygen molecule from fully saturated oxyhaemoglobin in sheep blood solutions. *Proc. Roy. Soc. Ser. B. Biol. Sci.* **143**: 310.
9. Imai, K. 1968. Oxygen equilibrium characteristics of abnormal hemoglobin Hiroshima ( $\alpha_2 \beta_2^{148 \text{ Asp}}$ ). *Arch. Biochem. Biophys.* **127**: 543.
10. Hill, A. V., and J. Barcroft. 1910. The nature of oxyhaemoglobin with special reference to its molecular weight. *J. Physiol. (London)*. **40**: 411.
11. Gibson, Q. H. 1970. The reaction of oxygen with hemoglobin and the kinetic basis of the effect of salt on binding of oxygen. *J. Biol. Chem.* **245**: 3285.
12. Antonini, E., and M. Brunori. 1970. Kinetics of the reaction of hemoglobin with ethylisocyanide. Interpretation of the results within a dimer model. *J. Biol. Chem.* **245**: 5412.
13. Nagel, R. L., H. M. Ranney, and L. L. Kucinskis. 1966. Tyrosine ionization in human carbon monoxide and deoxyhemoglobins. *Biochemistry*. **5**: 1934.
14. Moffat, J. K. 1971. Spin-labelled interpretation of electron paramagnetic resonance spectra based on x-ray analysis. *J. Mol. Biol.* **55**: 135.
15. Perutz, M. F. 1970. Stereochemistry of cooperative effects in haemoglobin. *Nature (London)*. **228**: 726.
16. Nagel, R. L., and Q. H. Gibson. 1971. The binding of hemoglobin to haptoglobin and its relation to subunit dissociation of hemoglobin. *J. Biol. Chem.* **246**: 69.

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