45. Oxygen-Equilibrium Characteristics of Hemoglobin Norfolk ($\alpha_2^{57\text{ASP}}\beta_2^A$)

By Shigeru FUJITA, Takashi IMAMURA, Yoshiro OHTA,
Masashi SEITA, Motosuke HANADA,
and Toshiyuki YANASE

First Department of Medicine, Faculty of Medicine,
Kyushu University

(Comm. by Tanemoto FURUHATA, M. J. A., March 12, 1969)

Recent studies of the structural and physicochemical characteristics of abnormal hemoglobins have contributed not only to our understanding of the pathophysiological mechanisms resulting from molecular abnormalities, but also to knowledge of the structure-function relationship of the normal hemoglobin molecule. A mutational substitution of a single amino acid in the hemoglobin molecule may alter the net surface charge of the molecule and thus lead to an electrophoretic mobility different from that of normal hemoglobin. In the majority of recognized abnormal hemoglobins this change in charge is not associated with hemoglobinopathic disorders. On the other hand the architectural and functional integrity of the molecule may be affected if the amino acid exchange has occurred at a strategic site in the polypeptide chain:¹⁾ thus the stability of molecular configuration or the functional property of heme group may be affected.

The present paper deals with the study of the oxygen-equilibrium characteristics of Hb Norfolk in which glycine is replaced by aspartic acid in the fifty-seventh residue position of the α -chain.^{2),3)} The structure-function relationship of the new aspartic acid side chain in the so-called 'basic center'⁴⁾ of the hemoglobin molecule is briefly discussed.

Materials and method. Blood used in this study was obtained with heparin as anticoagulant from the proband of the Nishiki-1 family already reported elsewhere.⁵⁾ Erythrocytes were washed four times with normal saline and lysed by the toluene method.⁶⁾ Thin-layer starch gel electrophoresis at pH 8.6 was performed as described previously.³⁾ Preparative starch block electrophoresis was carried out in a pH 8.6 barbital buffer according to Kunkel and Wallenius.⁷⁾ Unfractionated hemolysates and isolated samples of the mutant hemoglobin were dialysed against 0.1 M phosphate ($\text{KH}_2\text{PO}_4\text{-Na}_2\text{HPO}_4$) buffer of variable pH's for 24 hours at 4°C. The purity of each fraction was ascertained by thin-layer starch gel

electrophoresis, and hemoglobin concentration was measured spectrophotometrically after conversion to pyridine hemochromogen.⁸⁾ Each sample was diluted to 0.1% with the same buffer used for dialysis and examined by the following procedures:

Oxygen-equilibrium curves of hemoglobin were recorded automatically using the method of Imai *et al.*⁹⁾ In this procedure the oxygen partial pressure in the sample was measured with a Beckman 39065 polarographic oxygen sensor and the percentage of oxygenated hemoglobin was determined spectrophotometrically using monochromatic light at 564m μ . The curves obtained were continuous. Before and after measurement the visible absorption spectra of samples were checked using a Beckman DK-2 self-recording spectrophotometer.

Methemoglobin formation in the sample during the entire procedure was negligible, judging from the change in the spectra.

Results. The oxygen-equilibria of hemoglobins Norfolk and A were studied on the same day under identical *in vitro* conditions. Hemolysates treated by starch block electrophoresis did not show an increased propensity to methemoglobin formation during dialysis and oxygen-equilibrium studies under the conditions used. The oxygen-equilibrium curves of pure Hb A are shaped and positioned like those of the whole hemolysate from normal controls. The oxygen-equilibrium for isolated Hb Norfolk were in accord with the observations of unfractionated hemolysates of Hb Norfolk. As shown in Fig. 1, the oxygen affinity of Hb Norfolk is almost identical to

Fig. 1. The oxygen-equilibria of hemoglobins A and Norfolk isolated from the same hemolysate, at 20°C in 0.1 M phosphate at pHs 6.58, 7.06, and 7.41.

Fig. 2. The pH dependence of oxygen-equilibrium of hemoglobins A and Norfolk. The interaction constant, n , is shown for each set of data.

that of Hb A. The value of n in Hill's equation was derived for each hemoglobin solution (Fig. 2). Pure Hb Norfolk had an n of 2.76, while electrophoretically separated Hb A had an n of 2.82. A qualitative comparison of the Bohr effect in isolated Hb Norfolk and in Hb A is given in Fig. 2. Comparison of change in pH upon deoxy-

Fig. 3. The dependence of the oxygen-equilibrium of hemoglobins A and Norfolk on temperature, at pH 7.4 in 0.1 M phosphate at 15°C, 20°C and 25°C.

genation does not reveal any significant differences between Hb Norfolk and Hb A. The effect of temperature on oxygen affinity of Hb Norfolk is apparently identical with that of Hb A when examined at 15°C, 20°C and 25°C (Fig. 3).

These findings suggest that the physiological properties of Hb Norfolk are about the same as those of Hb A.

Discussion. Hemoglobin Norfolk is known to carry the amino acid substitution of glycine for aspartic acid in the 57th residue position of the α -chain of Hb A.^{2),3)} This variant does not show any hematological abnormalities and the spectrum is unaffected by this amino acid substitution. Oxygen-equilibrium characteristics presented in this paper show that the oxygen affinity and the Bohr effect of Hb Norfolk are about the same as those of Hb A. The heme-heme or interchain interaction as measured by n value in Hill's equation are virtually identical in Hb Norfolk and in Hb A.

There are three possible structure-function relations which might account for the impairment of heme function by the substituted side chain in an abnormal hemoglobin.¹⁰⁾ The first is analogous to the direct interionic effect or new bonding between the heme moiety and the side chain of amino acid residue. Hemoglobin M Boston and Hb M Saskatoon¹¹⁾ in which the distal histidine residue suffers replacement by tyrosine, show very low oxygen affinity, loss of sigmoid character of oxygen-equilibrium curves and absence of the Bohr effect.¹²⁾ In these abnormal methemoglobins, it is thought that an internal complex is formed between the heme iron and the new tyrosine residue, stabilizing the iron in the trivalent state. Another abnormal methemoglobin, Hb M Milwaukee 1 in which the substitution site is four amino acid residues away from the distal histidine of the β -chain,¹¹⁾ is known to have a new carboxyl side chain near the iron atom forming a strong ferric complex: yet in Hb Norfolk the carboxyl side chain of the new aspartic acid which is next to the distal histidine of the α -chain does not show direct interaction between the heme and substituted side chain, presumably because the COOH group is not close to the heme group in the tertiary configuration of the molecule. According to Perutz *et al.*,^{13),14)} heme makes contacts with helix E at E7 (distal histidine), E10 and E11.

Since the side chain of E6 (α 57) is directed away from the heme group and lies near the surface of the globin molecule, and the charged amino acid side chain, like that of aspartic acid, tends to protrude outwards from the surface of the molecule in an aqueous environment, the aspartic acid side chain E6 (α 57) in Hb Norfolk apparently is unable to make polar interaction with heme which is surrounded by non-polar residues in the crevice of the globin molecule.

The second mechanism is intrachain distortion of the normal relations between the helices and the heme group due to either steric hindrance, or new bonding relationships that result from a substituted side chain of an abnormal hemoglobin. This intrachain shift may alter the usual intrahelical hydrogen bond formations and cause instability of the molecular configuration which protects heme function. The third possible mechanism is an effect of the substituted side chain at a region of contact between non-polar residues of the α - and β -chains favoring the oxy-hemoglobin quaternary structure. In Hb Chesapeake,¹⁵⁾ Hb Yakima¹⁶⁾ and Hb Kempsey¹⁷⁾ which have a high oxygen affinity, and in Hb Kansas¹⁸⁾ which has a low oxygen affinity, a low heme-heme interaction has been demonstrated. The substitution sites of these abnormal hemoglobins with impaired heme function lie in the F and G helices and FG non-helical segment using the numbering system of Perutz.¹⁹⁾ The similar structural and functional alterations in these variants suggest a key structure-function role of the FG and G helix regions in determining normal oxygen affinity and heme-heme interactions.

In any event, the amino acid substitution in Hb Norfolk has produced no drastic effect either on the property of reversible combination with oxygen or on the heme-heme interaction that results in the normal sigmoid oxygen dissociation curve. These data contribute basic information concerning the structure-function relationship in the hemoglobin molecule.

Summary. Oxygen-equilibrium characteristics of Hb Norfolk ($\alpha_2^{57\Delta sp}\beta_2^A$) are shown to be identical with those of Hb A. The amino acid substitution occurred in the position E6 ($\alpha 57$) which is adjacent to the distal histidine residue in the primary structure of the mutant hemoglobin does not produce any measurable effect on the functional property of heme iron. The structure-function relationships of the substituted side chain are briefly discussed.

Acknowledgments. The authors wish to express their thanks to Professor M. Kotani, Dr. H. Morimoto and Dr. K. Imai of the Laboratory of Biophysics, Faculty of Engineering Science, Osaka University for contributing with both valuable advice and the arrangement of the instrument assembly for oxygen-equilibrium studies, and to Dr. H. B. Hamilton of Atomic Bomb Casualty Commission, Hiroshima, for his advice and careful reading of this paper.

References

- 1) Heller, P.: *Amer. J. Med.*, **41**, 799 (1966).
- 2) Baglioni, C.: *J. Biol. Chem.*, **237**, 69 (1962).

- 3) Imamura, T.: *Amer. J. Human Genetics*, **18**, 584 (1966).
- 4) Braunitzer, G., Hilshman, N., Rudloff, V., Hilse, K., Liebold, B., and Muller, R.: *Nature, London*, **190**, 480 (1961).
- 5) Imamura, T., Kosaka, K., Ohta, Y., Hanada, M., and Seita, M.: *Proc. Japan Acad.*, **43**, 166 (1967).
- 6) Drabkin, D. L.: *J. Biol. Chem.*, **164**, 703 (1946).
- 7) Kunkel, H. G., and Wallenius, G.: *Science*, **122**, 288 (1955).
- 8) Paul, K. G., Theorell, H., and Akeson, A.: *Acta Chem. Scand.*, **7**, 1284 (1953).
- 9) Imai, K.: *Biochim. Biophys. Acta* (in press).
- 10) Jones, R. T., Osgood, E. E., Brimhall, B., and Koler, R. D.: *J. Clin. Invest.*, **46**, 1840 (1967).
- 11) Gerald, P. S., and Efron, M. L.: *Proc. Nat. Acad. Sci.*, **47**, 1758 (1961).
- 12) Hayashi, A., Shimizu, A., Yamamura, Y., and Watari, H.: *Science*, **152**, 207 (1966).
- 13) Perutz, M. F., Kendrew, J. C., and Watson, H. C.: *J. Mol. Biol.*, **13**, 669 (1965).
- 14) Perutz, M. F., Muirhead, H., Cox, J. M., and Goaman, L. C. G.: *Nature, London*, **219**, 131 (1968).
- 15) Charache, S., Weatherall, D. J., and Clegg, J. B.: *J. Clin. Invest.*, **45**, 813 (1966).
- 16) Novy, M. J., Edwards, M. J., and Metcalfe, J.: *J. Clin. Invest.*, **46**, 1848 (1967).
- 17) Reed, C. S., Hampson, R., Gordon, S., Jones, R. T., Novy, M. J., Edwards, M. J., and Koler, R. D.: *Blood*, **31**, (1968).
- 18) Bonaventura, J., and Riggs, A.: *J. Biol. Chem.*, **243**, 980 (1968).
- 19) Perutz, M. F.: *J. Mol. Biol.*, **13**, 646 (1965).