Finally, the observation that fMet-tRNA exerts its inhibitory effect not only in the absence of a mRNA containing the appropriate AUG or GUG codons but in the presence of poly(U), which should interfere with the interaction of fMet-tRNA and the 30S ribosomal subunit, indicates that fMet-tRNA has strong affinity for the 30S subunit. Thus, even though the formation of a messengerless complex of the 30S subunit and fMet-tRNA has not been demonstrated directly, the participation of such a complex in the initiation of bacterial protein synthesis as in pathway 3 must be considered a possibility, especially with the 30S ribosomal subunits that are strongly inhibited by fMet-tRNA binding. On the other hand, the 30S subunits which interact weakly with fMet-tRNA, if they are not damaged ribosomes, may initiate protein synthesis by either pathway 2 or pathway 3, since other suggestive evidence obtained in this laboratory (Bernal *et al.*, 1974) rules out pathway 1.

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References

Baglioni, C. (1972), *Biochim. Biophys. Acta 287,* 189.
Bernal, S. D. Blumberg, B. M., and Nakamoto (1974), *Proc. Nat. Acad. Sci. U. S. 71,* 774.
Craven, G. R., Voynow, P., Hardy, S. J. S., and Kurland, C. G. (1969), *Biochemistry 8,* 2906.
Darnbrough, C., Legon, S., Hunt, T., and Jackson, R. (1973), *J. Mol. Biol. 76,* 379.
Ginzburg, I., Miskin, R., and Zamir, A. (1973), *J. Mol. Biol. 79,* 481.
Hamel, E., Koka, M., and Nakamoto, T. (1972), *J. Biol. Chem. 247,* 805.
Hershey, J. W. B., Dewey, K. F., and Thach, R. E. (1969), *Nature (London) 222,* 944.
Hershey, J. W. B., Remold-O'Donnell, E., Kolakofsky, D., Dewey, K. F., and Thach, R. E. (1971), *Methods Enzymol. 20C,* 235.
Iwasaki, K., Sabol, S., Wahba, A., and Ochoa, S. (1968), *Arch. Biochem. Biophys. 125,* 542.
Moore, P. B. (1973), *J. Mol. Biol. 79,* 615.
Nakamoto, T., and Hamel, E. (1968), *Proc. Nat. Acad. Sci. U. S. 59,* 238.
Nakamoto, T., and Kolakofsky, D. (1966), *Proc. Nat. Acad. Sci. U. S. 55,* 606.
Revel, M., Herzberg, M., and Greenspan, H. (1969), *Cold Spring Harbor Symp. Quant. Biol. 34,* 261.
Salas, M., Hille, M. B., Last, J. A., Wahba, A. J., and Ochoa, S. (1967), *Proc. Nat. Acad. Sci. U. S. 57,* 387.
Schreier, M., and Staehelin, T. (1973), *Nature (London), New Biol. 242,* 35.
Stanley, W. M., Salas, M., Wahba, A. J., and Ochoa, S. (1966), *Proc. Nat. Acad. Sci. U. S. 56,* 290.
Takanami, N., and Okamoto, T. (1963), *J. Mol. Biol. 7,* 323.
Van Duin, J., and Kurland, C. G. (1970), *J. Mol. Gen. Genet. 109,* 169.
Zamir, A., Miskin, R., and Elson, D. (1969), *FEBS (Fed. Eur. Biochem. Soc.) Lett. 3,* 85.

Nonequivalence of Chains in Hemoglobin Oxidation and Oxygen Binding. Effect of Organic Phosphates†

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ABSTRACT: Oxidation of Hb A is governed by at least two factors: (a) the intrinsic susceptibility to oxidation of chains which depend on their primary structure, and (b) the affinity of chains for oxygen or other ligands. 2,3-Diphosphoglycerate (2,3-DPG) stabilizes the deoxy conformation of hemoglobin which has a lower affinity for oxygen. This effect is maximal at acid and minimal at alkaline pH. Hemoglobin F_1 which does not bind organic phosphates does not show significant affinity changes in their presence (H. F. Bunn and R. W. Briehl (1970), *J. Clin. Invest. 49,* 1088). The present paper shows that: (a) the presence of 2,3-DPG in hemoglobin solutions increases the rate of autoxidation at acid pH where the binding is the most significant; this effect is correlated with the decrease in affinity for oxygen; (b) although the organic phosphates bind to non α chains, their effect is mostly exerted on the oxygen affinity of α chains.

The autoxidation rate of the four heme groups of hemoglobin depends on at least two factors: (1) the primary structure and its consequences on the chain concerned, and (2) the presence and type of ligand (Mansouri and Winterhalter, 1973).

Organic phosphates, notably 2,3-diphosphoglycerate (2,3-DPG),[1] are present inside the red cell in a concentration approximately equimolar to that of hemoglobin. The level of 2,3-DPG is an important regulator of oxygen affinity of the blood (Benesch *et al.*, 1968; Benesch and Benesch, 1969; Chanutin and Curnish, 1967; Rörth, 1968; Lo and Schimmel, 1969; Waldeck and Zander, 1969). It has been shown that in hypoxic states the level of 2,3-DPG increases with a concomitant decrease in oxygen affinity (Lenfant *et al.*, 1968; Eaton *et al.*, 1969).

Lowering the oxygen affinity of hemoglobin is achieved by a shift of the allosteric equilibrium in favor of the T (deoxy) con-

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[1] Abbreviation used is: 2,3-DPG, 2,3-diphosphoglycerate.

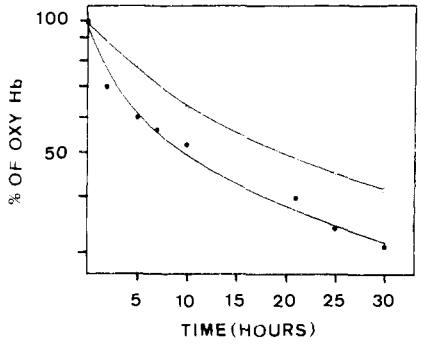

FIGURE 1: Autoxidation of Hb A at 37°, pH 6.8 in the presence (●) and absence (○) of 2,3-DPG.

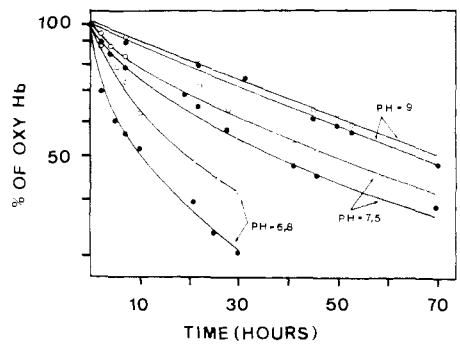

FIGURE 2: Autoxidation of Hb A at 37°, pH 6.8, 7.5, and 9 in the presence (●) and absence (○) of 2,3-DPG.

formation (Perutz, 1970). In a given solution, the presence of 2,3-DPG thus increases the concentration of molecules in the deoxy conformation with a low oxygen affinity. Hence the concentration of deoxy sites will increase. Since they are rapidly autoxidizable, the overall rate of oxidation will increase. This paper presents experimental evidence supporting the above reasoning.

Experimental Section

General. All procedures were performed at 4° unless otherwise stated. All buffers contained 10^{-4} M EDTA. 2,3-DPG was obtained from Calibiochem and was converted to its free acid form as follows. About 2 g of amberlite IR-120 (SERVA) was suspended in 8 ml of 2 N HCl and was stirred gently for 30 min. The amberlite was then packed in a small column (65 mm × 9 mm i.d.) and washed with twice distilled water to neutrality of the effluent; 6 ml of 0.01 M commercial 2,3-DPG was circulated through the above column at the rate of 4 ml/hr with a peristaltic pump for 24 hr. After this procedure the 2,3-DPG solution was ready for use. Inositol hexophosphate was obtained from Sigma. Both above compounds were of analytical grade.

Purification Procedures. Hemoglobin was purified by the method of Winterhalter and Huehns (1964). Hemoglobin A_{1C}[2] was separated from Hb A as described by Birchmeier *et al.* (1973). The fractions containing Hb A_{1C} (and Hb A and Hb F) were dialyzed against 0.01 M phosphate buffer (pH 6.8), applied to a CM-Sephadex column (18 × 200 mm) equilibrated with the above buffer, then eluted by a gradient of 1 l. of equilibrating buffer and 1 l. of 0.02 M Na_2HPO_4. Hb A_{1C} eluted as a separate peak with its maximum at pH 7.25, Hb F eluted with its peak at pH 7.35, and Hb A eluted with its peak at pH 7.47. Hemoglobin F was purified on a CM-Sephadex column as follows. About 15 ml of citrated cord blood was washed three times with normal saline solution. The cells were then lysed by the addition of 5 volumes of twice distilled water. After 20 min of centrifugation at 37 000*g* the supernatant was dialyzed against 0.01 M phosphate buffer (pH 6.8), then applied to a CM-Sephadex column (20 mm × 500 mm) equilibrated with the above buffer. Stepwise elution was achieved with 0.01 M phosphate buffer, starting at pH 6.8, then 7.0, 7.1, 7.2, 7.3, 7.4, and 7.5. Hemoglobin F was eluted at pH 7.3. The purity was checked by starch gel electrophoresis (Poulik, 1957). The electrophoretically pure fractions were then tested for alkali resistance by the method of Singer *et al.* (1951).

Hemoglobin F_1,[3] a minor component of cord blood, was purified in the same manner as Hb F. This Hb was eluted at pH 7.1 in very dilute form which had to be concentrated about 100 times. Alternatively, it was possible to obtain Hb F_1 by applying the cord hemolysates on a DEAE-Sephadex column and eluting stepwise by 0.1 pH unit with 0.05 M Tris-HCl buffer from pH 8.1 to pH 7.4. Hb F_1 was eluted at pH 7.6 (Jonxis and Huisman, 1968).

The purity of Hb F_1 was also checked by starch gel electrophoresis and alkali denaturation. This hemoglobin possesses the same mobility as that of Hb A on starch gel, but contrary to the latter it is alkali resistant.

Stripping Hemoglobin of Phosphates. All glassware was washed with phosphate-free soap prior to use. All hemoglobins to be stripped were first concentrated to about 1% solution and dialyzed against 0.05 M Tris-HCl (pH 7); 5 ml of Hb solution was passed through a column of G-25 Sephadex (20 mm × 500 mm) equilibrated with 0.1 M NaCl. The eluate was then dialyzed against the above Tris-HCl buffer of appropriate pH. The stripped hemoglobin solutions were tested for the presence of phosphate (Chen *et al.*, 1956).

Oxidation and Chain Separation. Procedures were the same as reported previously (Mansouri and Winterhalter, 1973). The only difference was replacement of phosphate buffer by 0.05 M Tris-HCl buffer of the appropriate pH.

Spectrophotometric Measurements. Spectra were obtained on a double beam spectrophotometer. Methemoglobin content was measured by the method of Dubowski (1964). Oxygen equilibria were done as described by Rossi-Fanelli and Antonini (1958).

Results

Oxidation of Deoxy- and Oxyhemoglobin by Potassium Ferricyanide, $K_3[Fe(CN)_6]$. The oxidation of deoxy- and oxyhemoglobin in a tonometer by various concentrations of potassium ferricyanide shows that the rate of the oxidation of deoxyhemoglobin is about two orders of magnitude higher than that of oxyhemoglobin (E. Di Iorio, A. Mansouri, and K. H. Win-

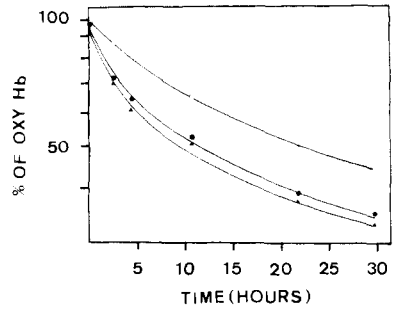

FIGURE 3: Autoxidation of Hb A in the absence (○) and in the presence of 2 molar excess (●) and 10 molar excess (▲) of 2,3-DPG.

[2] Hb A_{1C} = Hb A in which the N-terminal of β chain is blocked by a Schiff base (Holmquist and Schroeder, 1966; Bookchin and Gallop, 1968).

[3] Hb F_1 = Hb F in which the N-terminal of both γ chains are acetylated (Schroeder *et al.*, 1962; Huehns and Shooter, 1966).

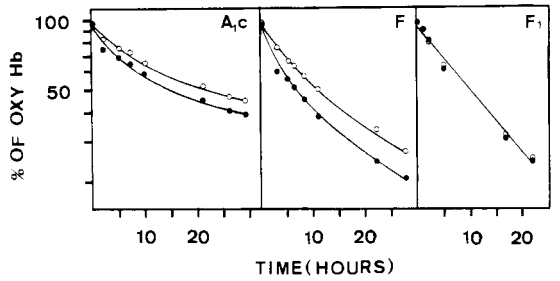

FIGURE 4: Autoxidation of Hb A_{1C}, F, and F_1 at 37°, pH 6.8 in the presence (●) and absence (○) of 2,3-DPG.

terhalter, 1973, unpublished data). Ligandization of hemoglobin thus sharply decreases the rate of oxidation.

The Effect of 2,3-DPG on the Rate of Autoxidation. Stripped hemoglobin in 0.05 M Tris-HCl (pH 6.8) was divided into two parts. To one part 2 mol excess of 2,3-DPG per mol of Hb was added and the pH readjusted. The samples were then incubated at 37° and the methemoglobin formation was measured as a function of time.

Figure 1 shows the results of such experiments. It is evident that the presence of phosphate markedly increases the rate of autoxidation at acid pH. It is also evident that the increase in the rate of autoxidation is more pronounced for the rapidly reacting component. The rate constants in 0.05 M Tris-HCl at pH 6.8 of rapid- and slow-reacting components, in the absence of 2,3-DPG, were 0.073 and 0.023 hr^{-1}, respectively, *vs.* 0.195 and 0.032 hr^{-1} in the presence of 2,3-DPG. The effect of 2,3-DPG is maximal at acid pH (6.8) and minimal at pH 9. Intermediate values were found at pH 7.5 (Figure 2).

Increasing the concentration of 2,3-DPG from 2 to 10 mol excess per mol of hemoglobin does not significantly increase the rate of autoxidation (Figure 3). The results of analogous experiments with hemoglobin A_{1C}, F, and F_1 are depicted in Figure 4. Similar to Hb A, the presence of 2,3-DPG increases the rate of autoxidation only at acid pH (Figure 4). The rate of autoxidation of the above hemoglobins is not dependent on the presence or absence of 2,3-DPG at pH 9. Analogously to 2,3-DPG, inositol hexophosphate increases the rate of autoxidation at acid pH (Figure 5).

Although it is known from previous work that the fast-reacting component represents the α chains (Mansouri and Winterhalter, 1973), chain separation was done on sample autoxidized to about 50% either in the presence or absence of 2,3-DPG. The per cent oxidation of the thus obtained chains was quantitated spectrophotometrically (Mansouri and Winterhalter, 1973). The α/β ratio in the presence of 2,3-DPG was 2.68, in its absence only 1.24, thus implying that 2,3-DPG increases the average concentration of α-deoxy sites more than the β-deoxy hemes.

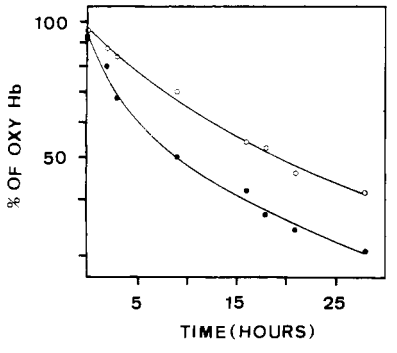

FIGURE 5: Autoxidation of Hb A at 37° pH 6.8 in the presence (●) and absence (○) of inositol hexophosphate.

TABLE I: Log $p(O_2)^{1/2}$ of Hb A, A_{1C}, and F in the Presence and Absence of 2,3-DPG at 20°; Hb Concentration, 1.25×10^{-4} M, 2,3-DPG Concentration, 10 M/M Hb.

Hb	pH	Log $p(O_2)^{1/2}$ (+) 2,3-DPG	Log $p(O_2)^{1/2}$ (−) 2,3-DPG	Difference of Log $p(O_2)^{1/2}$ in the Presence and Absence of of 2,3-DPG
A	6.8	1.72	0.76	0.96
A_{1C}	6.5	1.48	0.80	0.68
F	6.5	1.62	0.90	0.72

In order to see if the change of affinity in the presence or absence of 2,3-DPG correlates with the change in the rate of autoxidation, the affinity of Hb A, A_{1C}, and F in the presence or absence of phosphate was measured at acid pH. The results of these measurements are depicted in Table I.

Discussion

As previously reported (Mansouri and Winterhalter, 1973), the autoxidation of hemoglobin A has two components: (1) a rapidly oxidizing component which consists of α chains, and (2) a slowly oxidizing component, the β chains. Apparently, the rate of autoxidation of hemoglobin A is dependent on at least two factors: (a) the intrinsic susceptibility to oxidation on the basis of primary structure of polypeptide chain (α chains being more oxidizable than β chains); (b) the presence and nature of ligands. In Hb A—as α chains have a higher affinity for oxygen—decreasing the $p(O_2)$ increases the rate of autoxidation more for the β chains than for the α chains.

Organic phosphates such as 2,3-DPG are present in the red cell in large quantities. They lower the oxygen affinity of hemoglobin, thereby facilitating oxygen delivery to the tissues. The transformation of oxyhemoglobin to methemoglobin in 2,3-DPG depleted red cells is slower than when 2,3-DPG is present (Versmold *et al.*, 1973).

In the deoxy conformation the stereochemistry and charge distribution of the central cavity is such that the negatively charged 2,3-DPG molecule can bind (Perutz, 1970; Arnone, 1972). Four residues on each β chain are in the proper position to interact electrostatically with 2,3-DPG: the N-terminal amino group, histidine-2, lysine-82, and histidine-143. The effect of 2,3-DPG is most pronounced at low pH where the above mentioned groups are protonated (Figure 2). Binding of 2,3-DPG stabilizes hemoglobin in the deoxy conformation which has a lower O_2 affinity and thus increases the rate of autoxidation.

Assuming that the four groups play a similar role in the binding of 2,3-DPG, one would predict that the effect of 2,3-DPG in Hb A_{1C} having its N-terminal blocked and in Hb F having histidine-143 replaced by serine would be diminished by about one-fourth. In Hb F_1 the effect should be decreased by one-half, since its N-terminal is actylated and its histidine-143 is replaced by serine. It thus has only histidine-2 and lysine-82. However, Hb F_1 shows no effect at all, indicating that histidine-2 and lysine-82 are relatively unimportant in the binding of 2,3-DPG. Consequently, no appreciable effect on affinity for oxygen or the rate of autoxidation can be demonstrated. Hb F is more affected by 2,3-DPG than Hb A_{1C}. This could be due to nonequivalence of the α-amino and histidine-143 binding site.

Separation of chains after partial autoxidation in the presence and absence of 2,3-DPG reveals that the ratio of per cent oxidized α over oxidized β is larger when the hemoglobin solution is oxidized in the presence of 2,3-DPG.[4] This finding coupled with the marked increase of the rate constant of the fast-reacting component from 0.073 hr^{-1} in the absence of 2,3-DPG to 0.195 hr^{-1} in its presence brings strong evidence that, although the organic phosphates bind to non α chains, their overall effect on oxygen affinity and autoxidation rate is exerted mainly on the α chains. This is in accordance with the work of Olson and Gibson (1972) who used *n*-butyl isocyanide as a ligand. They concluded that the most striking effect of phosphate is a lowering of the ligand affinity of the α chains relative to that of β chains within the deoxy tetramer. The study of the effect of organic phosphates on oxygen affinity and on the rate of autoxidation of normal and fetal Hb, A_{1C}, and F_1 (minor constituents of adult and cord hemolysates, respectively) demonstrates that organic phosphates lower the oxygen affinity of hemoglobin through their binding. This decrease in affinity secondarily increases the autoxidation rate.

References

Arnone, A. (1972), *Nature (London) 234,* 146.

Benesch, R., and Benesch, R. E. (1969), *Nature (London) 221,* 618.

Benesch, R., Benesch, R. E., and Enoki, Y. (1968), *Proc. Nat. Acad Sci. U. S. 61,* 1102.

Birchmeier, W., Tuchschmid, P. E., and Winterhalter, K. H. (1973), *Biochemistry 12,* 3667.

Bookchin, R. M., and Gallop, P. M. (1968), *Biochem. Biophys. Res. Commun. 32,* 86.

Chanutin, A., and Curnish, P. R. (1967), *Arch. Biochem. 121,* 96.

Chen, P. S., Toribara, T. Y., and Warner, H. (1956), *Anal. Chem. 28,* 1786.

Dubowski, K. M. (1964), *in* Hemoglobin. Its Precursors and Metabolites, Sunderman, F. W., and Sunderman, F. W., Jr., Ed., Philadelphia, Pa., Lippincott, p 53.

Eaton, J. W., Brewer, G. J., and Grover, R. F. (1969), *J. Lab. Clin. Med. 73,* 603.

Holmquist, W. R., and Schroeder, W. A. (1966), *Biochemistry 5,* 2489.

Huehns, E. R., and Shooter, E. M. (1966), *Biochem. J. 101,* 852.

Jonxis, J. H. P., and Huisman, T. H. J. (1968), Abnormal Hemoglobins, 2nd ed., Oxford, Blackwell Scientific Publications, p. 49.

Lenfant, C., Torrance, J., English, E., Finch, C. A., Raynaf-arge, C., Ramos, J., and Faura, J. (1968), *J. Clin. Invest. 47,* 2652.

Lo, H. H., and Schimmel, P. R. (1969), *J. Biol. Chem. 244,* 5084.

Mansouri, A., and Winterhalter, K. H. (1973), *Biochemistry 12,* 4946.

Olson, J. S., and Gibson, Q. H. (1972), *J. Biol. Chem. 247,* 1713.

Perutz, M. F. (1970), *Nature (London) 228,* 726.

Poulik, M. D. (1957), *Nature (London) 180,* 1477.

Rörth, M. (1968), *Scand. J. Lab. Invest. 22,* 208.

Rossi-Fanelli, A., and Antonini, E. (1958), *Arch. Biophys. Biochem. 80,* 229.

Schroeder, W. A., Cua, J. T., Matsuda, G., and Fenninger, W. D. (1962), *Biochim. Biophys. Acta 63,* 532.

Singer, K., Chernoff, A. I., and Singer, L. (1951), *Blood 6,* 413.

Versmold, H., Ulmer, B., Riegel, K., and Betke, K. (1973), *Klin. Wochenschr. 51,* 93.

Waldeck, F., and Zander, R. (1969), *Klin. Wochenschr. 47,* 1068.

Winterhalter, K. H., and Huehns, E. R. (1964), *J. Biol. Chem. 239,* 3699.

[4] This ratio attains a value of about 10 when the autoxidation reaction takes place in 0.1 M phosphate buffer at acid pH (Mansouri and Winterhalter, 1973).