CYANATE AND HEMOGLOBIN-S: EFFECT OF CARBAMYLATION OF THE α AND β CHAIN α-AMINO GROUPS ON O_2 AFFINITY

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1. Introduction

Under appropriate experimental conditions cyanate carbamylates the four terminal α-amino groups of valine 1α and 1β of horse and human hemoglobin [1–3]. Recent investigations [4–8] have shown that, after oral or parenteral administration of this drug, a similar reaction occurs in vivo. Upon treatment with cyanate, erythrocytes of patients with sickle cell anemia, both in the circulating blood or in vitro, have a reduced tendency to sickling. This effect has been attributed to the increase in the oxygen affinity of hemoglobin S (HbS)* produced by irreversible carbamylation of the four terminal α-amino groups of the α and β chains of the molecule [9–10]. Kilmartin et al. [11] have recently shown for human hemoglobin that the increase in oxygen affinity upon combination with cyanate is mainly due to carbamylation of valines 1α. This finding has been confirmed also for HbS, but only for diluted, buffered solutions of this protein, in the absence of CO_2 and 2,3-DPG [12]. As these allosteric factors are the main factors in regulating the oxygen affinity of hemoglobin, it is important to assess the effect of cyanate in the treatment of SS-anemia, to study the changes in oxygen affinity of HbS, when cyanate is specifically reacted with the terminal α-NH_2 groups of the α and/or β chains. A relevant question immediately arises. Does cyanate affect the oxygen affinity of HbS simply by combining with the α-amino groups of the α chains or, as found in human hemoglobin, does it have additional effects by competing with CO_2 and 2,3-DPG for the α-amino groups of the β chains? The present report is an attempt to answer this question.

Abbreviations: HbS, the chromatographically purified main component of hemolysates from homozygous sickle cell individuals; $\alpha_2^C\beta_2^S$, the derivative of HbS where cyanate carbamylates the terminal α-amino group of α chains; $\alpha_2\beta_2^{SC}$, the derivative of HbS where cyanate carbamylates the terminal α-amino group of β chains; $\alpha_2^C\beta_2^{SC}$, the derivative of HbS where cyanate carbamylates the terminal α-amino group of the α and β chains; HbA, the chromatographically purified main component of hemolysates from normal individuals; 2,3-DPG, 2,3-diphosphoglycerate.

2. Materials and methods

The following cyanate derivatives were prepared: (1) $\alpha_2^C\beta_2^{SC}$ (i.e., the doubly-blocked derivative of HbS in which the terminal α-amino groups of the α and of the β chains were reacted with cyanate), and (2) $\alpha_2^C\beta_2^{sc}$ and $\alpha_2\beta_2^{sc}$ (in which cyanate carbamylates the α-amino group of the α chain and, respectively, of the β chain of HbS). Complete experimental details for the preparation of HbS and the various cyanate derivatives have been reported in detail elsewhere [13]

Due to the considerable work required to obtain the various derivatives and the short supply of HbS, it was essential to modify the method previously used to study the functional properties of the cyanate derivatives of HbA [11]. A much smaller cuvette was adapted to the equilibrating tonometer to accommodate micro samples (300 to 400 μl) of hemoglobin. The Hb samples were transferred from the tonometer by a microsyringe into a specially made microcuvette (∼ 100 μl capacity) which was fitted to a standard holder of a Beckman Acta III Spectrophotometer. The percent oxygen saturation was then obtained by

standard spectrophotometric techniques. Considerable care in the preparation of derivatives was necessary in order to minimize the formation of methemoglobin to less than 2 to 3%. A 7 to 8% oxidation of the protein can significantly affect the value of oxygen affinity.

3. Results

Fig.1 shows values of log P_{50} (i.e., the logarithm of the oxygen pressure required to give 50% oxygen saturation of hemoglobin) for $\alpha_2^C\beta_2^{SC}$, $\alpha_2^C\beta_2^S$, and $\alpha_2\beta_2^{SC}$, $\alpha_2\beta_2^S$. The experiments have been carried out in the absence of CO_2, 2,3-DPG, and buffers at a hemoglobin concentration of 5%, μ = 0.1 M (KCl). Fig.1 clearly

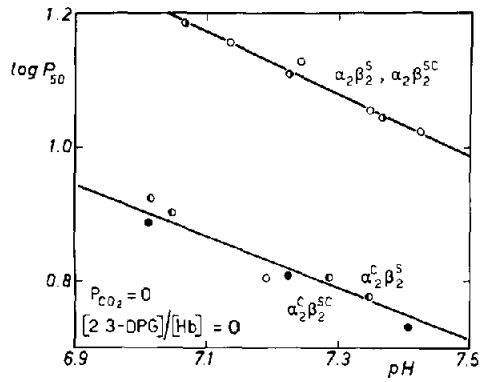

Fig.1. Log P_{50} values, in absence of 2,3-DPG and at zero P_{CO_2} for $\alpha_2^C\beta_2^S$ (•), $\alpha_2^C\beta_2^{SC}$ (•), $\alpha_2\beta_2^{SC}$ (•), and $\alpha_2\beta_2^S$ (○); Hb ∼ 4 mEq/litre.

shows that, under such conditions, only the cyanate reacted at the terminal α-amino groups of the α chain of HbS can increase the oxygen affinity of the molecule (this is shown by $\alpha_2^C\beta_2^S$ and $\alpha_2^C\beta_2^{SC}$ having the same oxygen affinity). Carbamylation of valine 1β, on the other hand, does not affect the oxygen affinity of HbS ($\alpha_2\beta_2^{SC}$ has the same oxygen affinity as the unreacted HbS molecule, $\alpha_2\beta_2^S$).

Fig.2 shows how the presence of physiological concentrations of CO_2 and 2,3-DPG can alter this picture. In this case it is interesting to note that (i) the *total* change in oxygen affinity ($\Delta \log P_{O_2}$ at constant pH) between $\alpha_2\beta_2^S$ and the doubly-blocked derivative $\alpha_2^C\beta_2^{SC}$ is 25 to 30% greater in the presence than in the absence of CO_2 and 2,3-DPG and (ii) a

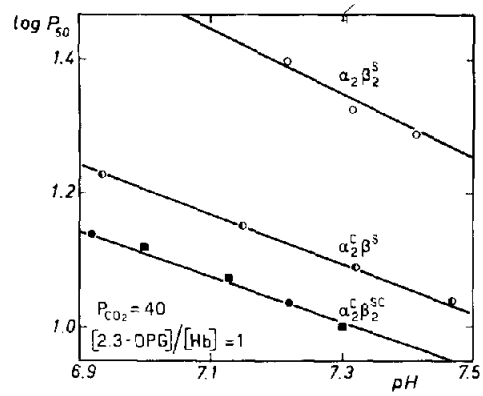

Fig.2. Log P_{50} values for $\alpha_2^C\beta_2^{SC}$ in the presence (•) of P_{CO_2} = 40 mm Hg; $\alpha_2^C\beta_2^S$ in the presence of P_{CO_2} = 40 mm Hg (•); $\alpha_2\beta_2^S$ in the presnece of P_{CO_2} = 40 mm Hg (○). 2,3-DPG concentration was 1.0 mol 2,3-DPG per hemoglobin tetramer for all the derivatives shown. The points (■) refer to $\alpha_2^C\beta_2^{SC}$ in absence of CO_2.

significant effect on oxygen affinity of carbamylation of valine 1β is now apparent ($\alpha_2^C\beta_2^S$ vs. $\alpha_2^C\beta_2^{SC}$).

4. Discussion

The data reported in fig.1, which are similar to those already found on HbA [11], can be explained in the framework of the allosteric theory of hemoglobin functions [14]. According to this theory, hemoglobin would exist in solution in two different conformations, R and T. The T form of the molecule has a low and the R form a high oxygen affinity. Transition from the T to the R form occurs upon breaking six hydrogen bonds which are know to stabilize the T form of the molecule. The combination of cyanate with the terminal α-amino group of the α chains would break the bond between this group and the C-terminal carboxyl group of Arg 141α [15], altering the allosteric equilibrium T $\rightleftarrows$ R towards the R form [16].

Additional effects of cyanate in presence of physiological concentrations of CO_2 and 2,3-DPG (as shown in fig.2) can be explained in a different way. CO_2 reacts with the four terminal α-amino groups of the α and of the β chains [3]. Both the carbamate bound at the α or the β chain is 'oxygen-linked.' i.e., less carbamate is bound by oxy- than by deoxyhemoglobin [17]. Alternatively, combination of CO_2 with the four α-amino groups decreases the oxygen affinity

of the molecule, but it is the CO_2 bound at the β chain that has the greatest effect [12]. On the other hand, 2,3-DPG reacts with a cluster of six positive charges around the central cavity of the deoxyhemoglobin molecule [8]. Two of these charges are contributed by the two-terminal α-amino groups of the β chains. Thus cyanate, CO_2, and 2,3-DPG compete for some of the same sites of the deoxy form of the molecule. If cyanate is bound, then CO_2 cannot bind at all with the same site on the α or β chain terminal α-amino groups, whereas 2,3-DPG can still bind at the β-chain site but with a lower affinity [12].

At a physiological level the effect of cyanate on the oxygen affinity of HbS under physiological conditions is quite significant. Fig.2 shows that the P_{50} of concentrated HbS (in absence of sickling), at pH 7.2 (i.e. at the Ph value near to that found in the interior of the erythrocyte in vivo), is approx. 25 mM hg. Complete carbamylation of the two α-amino groups both on the α and β chains decreases the P_{50} to approx. 14 mm Hg. The data reported in fig.1 and 2 shows that such a large shift is due to two combined effects: (i) the binding of cyanate at the terminal α-amino groups of the α chains, and (ii) the simultaneous complete disappearance of the effect of CO_2 and the decrease of the effect of 2,3-DPG on the oxygen affinity of HbS.

The experiments reported in figs.1 and 2 have been carried out at a moderately high protein concentration (5%), but in absence of sickling. The next step would be the study of the effect of cyanate bound at the α or at the β chains α -amino groups on the oxygen affinity of HbS under aggregating conditions. Such a study would clearly be a major undertaking as it would require the development of suitable techniques for the measurement of oxygen saturation in gels of HbS.

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References

[1] Kilmartin, J. V. and Rossi-Bernardi, L. (1969) in: CO_2: Chemical, Biochemical and Physiological Aspects, (R. E. Forster, J. T. Edsall, A. B. Otis and F. J. W. Roughton, eds.) pp. 73–81 U.S. Government Printing Office, NASA No. SP-188, Washington, D. C.
[2] Kilmartin, J. V. and Rossi-Bernardi, L. (1969) Nature 222, 1243–1246.
[3] Kilmartin, J. V. and Rossi-Bernardi, L. (1971) Biochem. J. 124, 31–45.
[4] Cerami, A. and Manning, J. M. (1971) Proc. Natl. Acad. Sci. 68, 1180–1183.
[5] Gillette, P. N., Manning, J. M. and Cerami, A. (1971) Proc. Natl. Acad. Sci. 68, 2791–2793.
[6] De Furia, F. G., Miller, D. R., Cerami, A. and Manning, J. M. (1972) J. Clin. Invest. 51, 566–573.
[7] Manning, J. M., Cerami, A., Gillette, P. N., De Furia, F. G. and Miller, D. R. (1972) in: Advances in Experimental Medicine and Biology, (G, J. Brewer, ed.) Vol. 28, pp. 253–260 Plenum Press, New York.
[8] Gillette, P. N., Peterson, C. M., Manning, J. M. and Cerami, A. (1972) ibid. pp. 261–271 Plenum Press, New York.
[9] May, A., Bellingham, A. J., Huehns, E. R. and Beaven, G. H. (1972) Lancet 658–661.
[10] May, A. and Huenhs, E. R. (1974) Trans. Roy. Soc. Trsp. Med. Hyg. 68, 85–91.
[11] Kilmartin, J. V., Fogg, J., Luzzanna, M. and Rossi-Bernardi, L. (1973) J. Biol. Chem. 248, 7039–7043.
[12] Nigen, A. M., Njikam, N., Lee, C. K. and Manning, J. M. (1974) J. Biol. Chem. 249, 661–6616.
[13] Perrella, M., Bresciani, D., Guglielmo, G., Russo, V., Rossi, F., Rossi-Bernardi, L., Righetti, P. G. and Kilmartin, J. V. in: Third International Symposium on Red Cell Metabolism and Function, Ann Arbor, 1974 (in press).
[14] Monod, J., Wyman, J. and Changeux, J. P. (1965) J. Mol. Biol. 12, 88–118.
[15] Perutz, M. F. (1970) Nature 228, 726–739.
[16] Kilmartin, J. V., Imai, K. and Jones, R. T. in: Third International Symposium on Red Cell Metabolism and Function, Ann Arbor, 1974 (in press).
[17] Becklake, M. R., Griffiths, S. B., McGregor, M., Goldman, H. I. and Schreve, J. P. (1955) J. Clin. Invest. 34, 751–755.
[18] Rossi-Bernardi, L. and Roughton, F. J. W. (1967) J. Physiol. 189, 1–29.
[19] Arnone, A. (1972) Nature 237, 146–149.