Temperature Dependence Study of the Reaction of Human Oxyhemoglobin "A" with 5,5' -Dithiobis (2-Nitrobenzoic Acid).

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Summary: The temperature-dependence study of the reaction of human oxyhemoglobin A (O_2HbA) with 55'-, dithiobis(2-nitrobenzoic acid), DTNB, as a function of pH has been studied. The quantitative analysis of the pH dependence of the apparent second order rate constant shows that two ionizable groups are electrostatically linked to the reaction. Their pKa values are 5.5 and 8.7. These values are assigned to His HC_3 (146) β and to the Cys F9 (93) β sulphydryl The intrinsic activation parameters for these ionizable groups have been determined using the Arrhenius and Eyring theories. The intrinsic activation entropies or His HC_3 (146) β and Cys F9 (93) β have confirmed that the two ionizable groups are electrostatically linked. Negative value of activation entropy for Cys F9 (93) β shows that there is an increase in the polarity around the reactive center. Large and positive intrinsic activation entropy of His HC (146) β further confirm that the presence of salt bridge between His HC3 (146) β and Asp FG 1 (94) β plays a major role in the reaction. The variation of apparent activation parameters as a function of pH could be attributed to the effect of salt bridges, hydrogen bonding, van der Waals forces and hydrophobicity of oxyhemoglobin A.

Introduction

Human oxyhemoglobin A has two titrable sulphydryl groups in the native form and six after denaturation [1]. The six sulphydryl groups are located at the following positions: Cys G11(104)α, Cys F9(93) β and Cys G14(112) β [1]. The extra four are thus masked in the native structure and may be concerned in the binding which holds together the two α and β chains in the tetramer [1]. Hemoglobin sulphydryl groups can react with oxidizing, electrophilic or NO (nitrogen monoxide) donating agents. The reactivity of titrable groups is independent of hemoglobin quaternary structure and mainly dependent on their low pKa in accompanying with stabilization of sulphydryl anion by a hydrogen bonding and also the exposure of these groups to the solvent and their accessibility to attack by reactants [2-3]. The reactivity of untitrable sulphydryl groups is dependents on hemoglobin quaternary structure. Hemoglobin from different species or different kinds of human hemoglobin (HbA, HbF, HbH, HbS, and so on) and even different derivatives of adult hemoglobin (carbomonoxy, acomet, azidomet oxy) have somewhat different structures and shows different sulphydryl reactivity [4,6]. In the folded structures, sulphydryl groups are hindered and the reactivity of these groups are obliged, as in human adult hemoglobin. [4-6]. Organic phosphates such as inositol hexaphosphate (IHP) or 2,3-diphosphoglycerate (DPO) can bind to HbA and changes its structure to the tens or folded structure, decreased the sulphydryl reactivity [7-8]. Hemoglobin with loosed structure as HbH or by using a denaturant to opening the hemoglobin structure will increase the reactivity of sulphydryl groups. The reaction of animal oxyhemoglobin with DTNB have shown that His HC_3 (146) β is electrostatically linked to the reactive Cys F9 (93) β [5]. The reaction of O_2HbA with 2,2'-dithiobispyridine reveals that the pK of Cys F9 (93) β is responsible for the reaction [9]. It has been suggested that the reactivity of the Cys F9 (93) β sulphydryl depends on two factors: the conformation of the sulphydryl group and the electrostatic effects of the charged ionizable groups on the protein [10]. 2,2'-dithiobispyridine is an uncharged sulphydryl reagent therefore its reaction will be sensitive to the conformation of the sulphydryl group or changes in the pK of the sulphydryl group rather than the electrostatic effects of the charged ionizable groups on the protein [10] while DTNB as a charged sulphydryl reagent, its reaction will be sensitive to both factors and the evaluation of these factors can be possible simultaneously by this reagent. In the previous works the reactivity of sulphydryl groups have been studied as a function of pH and at

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specified temperature [5,10-16] but there is no reports on the reaction of human oxyhemoglobin with sulphydryl reagents as a function of pH and temperature, so we have undertaken a comprehensive temperature dependence study of the reaction of oxyhemoglobin A with DTNB as a function of pH, at ionic strength of 0.05 M to determine which ionizable groups of oxyhemoglobin control the reactivity of the sulphydryl group and how these ionizable groups effect to this reactivity and also to determine the apparent and intrinsic activation parameters for the overall reaction and these ionizable groups, respectively. These kinetic parameters will be used to explain the effect of salt bridges, hydrogen bonding, van der Waals forces and hydrophobicity influence on the reaction.

Results and Discussion

Kinetics

The pH -dependence of the second-order rate constant (k) for the reaction of 0_2HbA with DTNB at different temperatures are shown in Fig.l. The titration of 02 HbA with DTNB is limited to pH 9 because the rate of hydrolysis of disulphide bonds increases markedly above pH 9. In this paper, we focused our attention on the pKa of the ionizable groups and the activation parameters for the sulphydryl reaction. The pH profiles in Fig.l. can be explained in two ways either by the fractional population of the thiolate anion of the sulphydryl group or the effect of salt bridges, peptide-peptide hydrogen bonding and hydrophobicity of the sulphydryl group. It has been established that there is a salt bridge between His HC3 (146) β and Asp FGl (94) β in the T-state hemoglobin [17]. This salt bridge is bond to retard the rate of reaction [18,19] at low pH which hemoglobin is more exist in the T - state. This equation:

$$k_{app} = k_1 \frac{K_1}{K_1 + [H^+]} + k_2 \frac{K_2}{K_2 + [H^+]} \qquad (1)$$

was used to fit our experimental data. In equation (1), k_1 is the limiting apparent second-order rate constant at high pH for the DTNB reaction when the reactivity of the Cys F9 (93) β sulphydryl is linked to the ionization of His HC_3 (146) β with ionization constant K_1; k_2 is the limiting apparent second order rate constant at high pH when the sulphydryl reactivity is linked to the ionization of Cys F9 (93) β with ionization constant K2. The first fractional term is the fraction of the neutral form of histidine, while the second fractional term is the fraction of the thiol anion form of the sulphydryl. The best-fit parameters are reported in Table I, which shows that the pKa values for His HC_3 (146) β and Cys F9 (93) β are almost the same for all temperatures studied. The result shows that the pKa values of these ionizable groups is temperature independent.

Table I. Reaction of DTNB with O_2HbA at different temperatures. Best -fit parameters used to fit the data of Fig.1 in to equation (1).

Table I

Temp.	pK_1	pK_2	$k_1(M^{-1}S^{-1})$	$k_2(M^{-1}S^{-1})$
10°C	5.5	8.7	7.0	10.53
15 °C	5.3	8.8	21.26	26.78
25 °C	5.5	8.7	49.66	55.34
35 °C	5.5	8.7	69.41	72.92

The intrinsic second order rates increases with increase in temperature.

In Fig. 1 at low temperatures and pH, decrease in the reaction rate could be attributed to high fractional proportion of the unreactive Cys F9 (93) β .The good fit of our ". experimental data corresponds to the previous fit reported in the earlier papers [5,12].

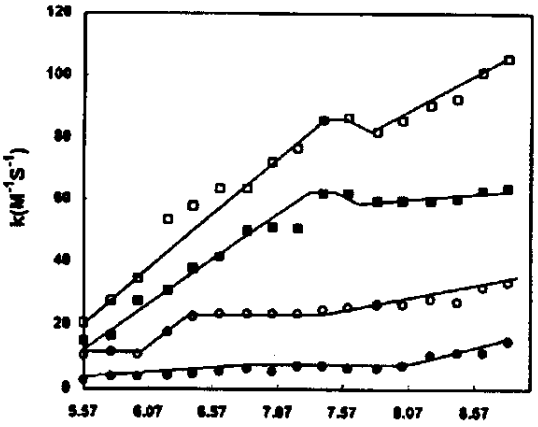

Fig.l.: Reaction of 5, 5'- dithiobis {2-nitrobenzoic acid) with the Cys F9 {93)β of oxyhemoglobin A.Dependence of $k_{(app)}$, the apparent second -order rate constant, on pH. The lines through the points are theoretical best -fit lines calculated with equation (1) of the text. I= 0.05 M. F •, 10°C; opened circle, 15 °C, ■, 25°C, opened square 35°C, Standard error = 5%

Temperature -dependence Study

The second-order rate constants obey the Arrhenius law to a good approximation [20], and the

activation energy was calculated at each pH. The Arrhenius law can be written as:

$$k = Ae^{-E/RT} \qquad (2)$$

where R is the gas constant, T the absolute temperature, E the energy of activation, and A the frequency factor. Figs 2 and 3 show the linear regression plots of lnk against 1/T. The slope obtained at each pH was used to calculate the value of E. This method was used to calculate the intrinsic activation energies of these ionizable groups. In terms of activated- complex theory [21], the equation in the case of reactions in solution can be written as:

$$\ln k = \ln\frac{K_t}{h} + \frac{\Delta S^*}{R} - \frac{\Delta H^*}{RT} \qquad (3)$$

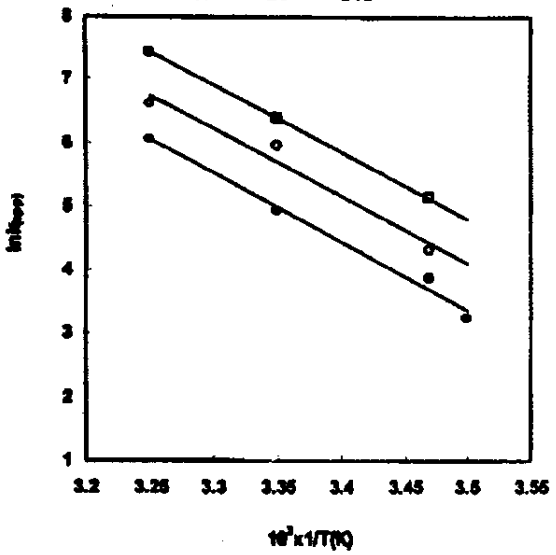

Fig.2: Arrhenius plot of lnk (app) against 1/T •, pH 6.2; opened circle, pH 7.0; and opened square, pH 8.8.

Where K is the Boltzmann constant and h is Planks constant. ΔS^* (activation entropy) was calculated from the ordinate intercept from the plots of lnk against 1/T. ΔH^* (activation enthalpy) was calculated using this equation [22].

$$\Delta E = \Delta H^* + RT \qquad (4)$$

Fig. 2 shows plot of linear regression of lnk against $1/T$ at different pH while Fig. 3 shows the linear regression plots of lnk against 1/T for the two ionizable groups. The intrinsic activation parameters for these ionizable groups were calculated using equations [5,9,12]. Table II shows the intrinsic activation parameters for these groups. Table II

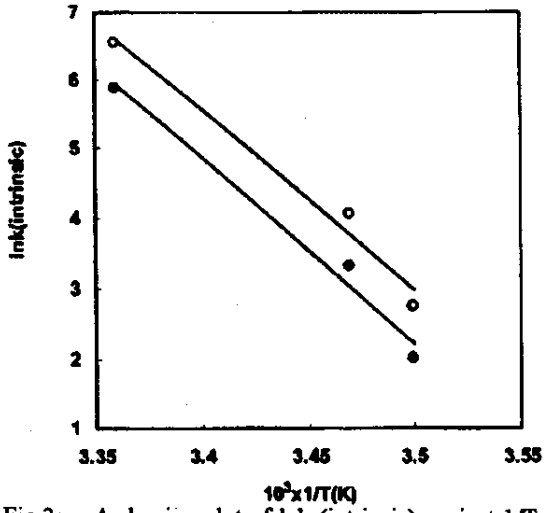

Fig.3: Arrhenius plot of lnk (intrinsic) against 1/T. •, His HC_3(143) and opened circle, Cys F9(93)

Table II:The intrinsic activation parameters of His HC_3 (146) β and Cys F9 (93) β of oxyhemoglobin A.

	E (kJ/mol)	ΔH^* (kJ/mol)	ΔS^* ($Jmol^{-1}K^{-1}$)
His HC3(146) β	91.94	89.47	+97.27
Cys F9(93) β	54.91	52.44	-29.18

Energies and Enthalpies of Activation:

Fig. 4 shows plots of E and ΔH^* against pH. This systematic variation has contributed to the understanding of the sulphydryl reactivities of hemoglobin that the tertiary structure of hemoglobin and the conformation of Cys F9 (93) β in the native form playa major role in the reaction. The values of E and ΔH^* increases as the pH increases within the pH range of 5.6 to 6.0. It shows that the hydrophobic side-chain residue is less exposed to the polar solvent medium, decrease in the rupture of salt bridges, decrease in peptide-peptide hydrogen bond breaking and increase in van der Waals forces [19]. This reason can be advanced for the variation in the pH range of 7.0 to 7.6. In between pH 6.0 and 6.8 and later pH 7.8 and 8.8, the values or E and ΔH^* decreases which show that the hydrophobic side chain of Cys F9 (93) β is more exposed to the polar solvent medium increase in the rupturing if the salt bridges, peptide-peptide hydrogen bond breaking and decrease in van der Waals. These characterities are associated with the reactive Cys F9 (93) β S^- which is electrostatically linked to His HC_3 (146) β. Intrinsic activation energy and enthalpy of Cys F9 (93) β are less when compared with that of His HC_3 (146)~

which shows that these ionizable groups are electrostatically linked.

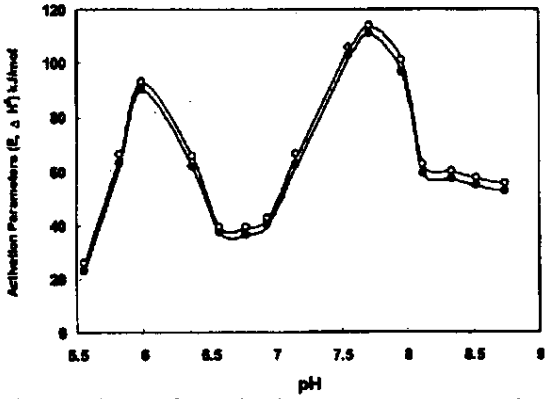

Fig.4: Plot of activation parameters against pH.Opened circle, activation energy (E}; •, activation enthalpy (ΔH^*).

Entropies of Activation

ΔS^* values are positive and negative over the pH range of study (see Fig.5). The variation reflects the difference in the number and character of the translational, rotational, and vibrational degrees of freedom between the transition state and reactants. The values of ΔS^* increases within the pH range of 5.6 to 6.0. This phenomenon can be attributed to the fact that the Cys F9 (93) β is not exposed to the solvent or the presence of salt bridge between His HC3 (146) β and Asp FG 1 (94) β at low pH. The presence of salt bridge must have hindered the degree of freedom of Cys F9 (93) β which subsequently retards the rate of reaction. From pH 6.8 to 6.0 and 8.8 to 7.8, ΔS^* increases. Increase in ΔS^* is synonymous with the fact that hemoglobin assumes a more open conformation when it reacts with DTNB to form a complex. Increase in ΔS^* might be due to the hydrogen bond breaking so that the thiolate anion possesses more degree of freedom during complexation. The interpretation of entropies of activation in terms of solvent effect is as follows. During the course of reaction there may be polarity changes that will result in either an increase or decrease in solvent binding. If charges are formed during a reaction, for example, the solvent will be bound with the charged group which will result into a loss of entropy. If, on the other hand, charges are neutralized during the reaction, there will be a release of solvent molecules and a corresponding gain of entropy [21-22]. Increase in ΔS^* within the pH range of 6.8 to 6.0 and subsequent pH 8.7 to 7.8, this variation can be attributed to the neutralization of charges during complexation which results into the release of solvent molecules. In these pH range, ΔS^* values are negative which further confirms that polarity increases at the reaction center. Electrostatic interaction between His HC_3 (146) β^+ - *S* and Cys F9 (93) β - *S* leads to negative entropy changes while the interaction between His HC3 (146) β^+ - *SH* and Cys F9 (93) β - *SH* leads to positive entropy changes. Figs 4 and 5 within the pH range of 7.0 to 7.6, the values of E and ΔS^* are positive. This variation shows that the thiol group is in the neutral form. If a process has a very high energy of activation and occurs at an appreciable speed at ordinary temperature, it must have a large positive entropy of activation. Positive E and ΔS^* might be associated with the hemoglobin conformation and exposure of the thiol group. When ΔH^* and ΔS^* (see Fig 4 and 5) are positive within the pH range of 7.0 to 7.6, the primary forces responsible for an increase should either be hydrogen bonding or van der Waals or both. The intrinsic ΔS^* of His HC_3 (146) β and Cys F9 (93) β shows that they are electrostically linked at the negative ΔS^* of Cys F9 (93) β shows that the thiol group is more exposed to the solvent medium. Conclusively, solvent effect plays a vital role in sulphydryl reactivity of oxyhemoglobin.

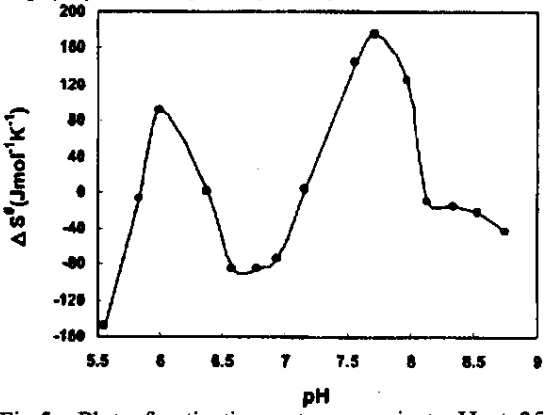

Fig.5: Plot of activation entropy against pH at 25 °C.

Experimental

Blood from normal human donors was obtained from the blood bank, Tehran hospital, Tehran, Iran. Hemoglobin A was prepared according to normal laboratory procedure [23]. DTNB was purchased from Sigma Chemical Company and was used without further treatment. DTNB solution was prepared by dissolving 0.9007 g of DTNB in phosphate buffer (pH 7.6, I=0.05 M) in a 250 ml

volumetric flask, and the mixture was magnetically stirred for four days. The solution was then filtered and its concentration was determined spectrophotometrically by measuring the optical density at 412nm after reacting it with excess mercaptoethanol. A molar extinction coefficient of 13,600 M^{-1} cm^{-1} was assumed for 3-carboxylato-4-nitro thiophenolate [18].

The kinetics were studied on a Shimadzu temperature controlled cell Holder TCC-240A computerized double beam UV-160 spectrophotometer at 10°C, 15°C, 25°C and 35°C in variation of 0.1°C. At low temperatures, the spectrophotometer was connected to dry air to prevent condensation of vapor on the cuvette during the measurement. Solutions of hemoglobin (10μM) were prepared in phosphate buffers (pH 5.6 to 8.0) and borate buffers (pH > 8.0), each of total ionic strength of 0.05 M. The solutions were allowed to equilibrate at the desired temperature. A 3 ml aliquot of each solution was pipetted into a 1 cm cuvette which was subsequently placed in the cell compartment of the spectrophotometer. A few microliters of DTNB of known concentration was measured with a Finn pipette into a glass rod shaped in a shallow spoon form. The rod was used to add the DTNB and to stir the DTNB-hemoglobin mixture. The absorbance of the mixture was recorded as a function of time at a wavelength of 412nm by the spectrophotometer. Each kinetic run was repeated twice under identical experimental conditions. Apparent second order rate constants $k_{(app)}$, were calculated with the second order rate equation. The extinction coefficients used in the calculation at 25 °C which have been reported before as a function of pH for 5-thio-2-nitro- benzoate [24], the product of the DTNB reaction. The molar extinction coefficients were redetermined for 10°C, 15°C, 25°C and 35°C. The concentration of DTNB in the cuvette ranges between 30 and 50 μM. The pH of the reaction mixture was determined using Beckman $\phi TM^{90}pH$ | ISE meter.

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