Evidence for a kinetic heterogeneity in ligand binding to R-state haemoglobin Kempsey [Asp-G1(99) β $\rightarrow$ Asn]

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Thermodynamic and kinetic properties of O₂ and CO binding to haemoglobin (Hb) Kempsey [Asp-G1(99) β $\rightarrow$ Asn] were investigated and the activation parameters for the two ligands were determined. At every temperature the O₂-binding isotherms display a weak co-operativity, n ranging between 1.1 and 1.2, and dissociation kinetics show a single-exponential behaviour. O₂-binding kinetics were studied at 25 °C by temperature jump and are characterized at each saturation (from $\bar{Y} = 0.31$ to $\bar{Y} = 1.0$) by two processes, a fast bimolecular one and a slow monomolecular one ($\tau^{-1} = 20 \text{ s}^{-1}$), which contributes to approx. 30% of the whole relaxation amplitude at every $\bar{Y}$. CO-binding kinetics to Hb Kempsey were followed at several temperatures by flash photolysis and stopped flow. The process is biphasic, as reported elsewhere [Bunn, Wohl, Bradley, Cooley & Gibson (1974) *J. Biol. Chem.* **249**, 7402–7409], and the relative contributions of the two bimolecular rates to the whole process are only slightly affected by temperature. On taking account for the fraction of dimers at every protein concentration, the slow phase corresponds to approx. 50% of the ligand binding to tetramers. Correlation of these results with previous spectroscopic data leads to the hypothesis that the biphasic time course of CO binding may be attributed to α/β heterogeneity of the R-state of tetrameric Hb Kempsey.

INTRODUCTION

The formulation of a molecular mechanism for ligands reacting with human haemoglobin has taken great advantage of studies on mutant haemoglobins characterized by amino acid substitutions at sites playing an important role in the control of ligand binding. Among these, as an example, studies carried out on mutant molecules in which residues on the distal side of the haem pocket are substituted, such as Hb Zürich (Giacometti *et al.*, 1980) and Hb Sydney (Tucker *et al.*, 1978), have clarified the key role played by these residues in the kinetics of ligand binding. Moreover, those carrying substitution at the $\alpha_1\beta_2$ and $\alpha_2\beta_1$ intersubunit contacts have been considered to be particularly interesting, since these interfaces have been convincingly suggested to play a critical role in the modulation of the relative stabilization of different quaternary states and thus on co-operativity (Perutz *et al.*, 1974; Pettigrew *et al.*, 1982). As a consequence of these structural perturbations, in such a class of mutant haemoglobins the allosteric equilibrium between the two quaternary conformations is dramatically affected, usually favouring the state whose stabilization energy is not altered by the amino acid replacement (Pettigrew *et al.*, 1982).

Hb Kempsey [Asp-G1(99) β $\rightarrow$ Asn] is characterized by a substitution that is involved in the stabilization of the T quaternary structure. Previous work (Lindstrom *et al.*, 1973; Bunn *et al.*, 1974; Nagai *et al.*, 1975) has indicated that Hb Kempsey maintains, in the absence of organic polyphosphates, an R-like conformation even in the fully deoxygenated derivative. This view seems to be supported mainly by the O₂-binding isotherms, which display a Hill

coefficient only slightly above unity, and a high O₂ affinity (Bunn *et al.*, 1974). Further support comes from O₂-pulse kinetic measurements (Gibson, 1973), in which an O₂ dissociation rate constant very similar to that of R-state human Hb A was observed in Hb Kempsey even at low degrees of saturation (Bunn *et al.*, 1974). Finally, it has been reported that the tetramer–dimer dissociation constant of deoxy-Hb Kempsey is very similar to that of the fully oxygenated protein (Pettigrew *et al.*, 1982).

However, the kinetics of CO binding have clearly shown the existence of heterogeneity, which has remained unexplained, and which may be of significance if Hb Kempsey is taken as a model for R-state deoxy-Hb. The present paper addresses to this point, and reports an extended kinetic and thermodynamic comparison between O₂ and CO binding to this abnormal haemoglobin.

MATERIALS AND METHODS

Blood of carriers of Hb Kempsey was generously given by Professor R. Carrell (Christchurch Hospital, Christchurch, New Zealand); Hb Kempsey was purified from Hb A in the 'stripped' form by the procedure of Bunn *et al.* (1974) and kept frozen in the CO form. Before use, samples were converted into the oxygenated derivative by exposure to a continuous strong light in the presence of O₂; the oxygenation was monitored spectrophotometrically. Throughout this work the buffer employed was 0.2 M-Bistris/HCl.

O₂-binding kinetics were followed either with a temperature-jump apparatus (Messanlagen Gesellschaft, Göttingen, Germany) (Brunori *et al.*, 1981) or with a stopped flow apparatus (Aminco Corp., Silver Spring,

Abbreviation used: Hb, haemoglobin.

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MD, U.S.A.) with use of the dithionite method (Hartridge & Roughton, 1923).

CO-binding kinetics were followed either by flash photolysis at 436 nm (Brunori & Giacometti, 1981) or by stopped flow with a Gibson-Durrum instrument (at 375 nm). CO dissociation kinetics were followed in a Cary 219 spectrophotometer by mixing CO-saturated Hb Kempsey (no free CO) with an excess of deoxymicroperoxidase (Sigma Chemical Co.). The rate of formation of CO-microperoxidase, corresponding to the dissociation of CO from Hb Kempsey, was detected as a decrease in A_{550} (Sharma *et al.*, 1976).

O₂-binding isotherms were obtained with a Hemoscan O₂-dissociation apparatus (Aminco Corp.) or by the tonometric method of Rossi Fanelli & Antonini (1958). In view of the very weak co-operativity, the free energy of O₂ binding was calculated according to the relationship $\Delta G = RT \cdot \ln c_1$, and c_1 was obtained from half-saturation O₂ pressure by using Henry's law (270 μM -O₂ in solution at atmospheric pressure) and $\Delta H = -12.1 \text{ kJ/mol}$ (-2.9 kcal/mol) for O₂ solubility.

Similarly, $\Delta H = R[d(\ln c_1)/d(1/T)]$ and $\Delta S = (\Delta H - \Delta G)/T$.

RESULTS

O₂-binding equilibria

O₂-binding isotherms of Hb Kempsey at several temperatures (between 15.2 °C and 39.6 °C) display a Hill coefficient $n = 1.1$ – 1.25 (Fig. 1). These values are similar to those previously reported (Bunn *et al.*, 1974), although in our case a 2-fold lower O₂ affinity is observed. At the moment we have no explanation for this difference, which has been also observed by others (Matsukawa *et al.*, 1981), even though the discrepancy itself is not very severe. The overall enthalpy of O₂ binding

Fig. 1. Hill plot of O₂-binding isotherms of Hb Kempsey at different temperatures

Temperature: ●, 15 °C; ▲, 19.9 °C; △, 24.6 °C; ○, 29.3 °C; ■, 34.9 °C; □, 39.6 °C. Buffer: 0.2 M-Bistris/HCl, pH 7.0. Inset: temperature-dependence of $K (= 1/c_1)$. The continuous line represents the linear least-squares fitting of the data.

Fig. 2. O₂-binding kinetics to Hb Kempsey by temperature jump

The reciprocal of the relaxation time for the fast kinetic component is plotted against the concentration of the free reacting species. Buffer: 0.2 M-Bistris/HCl, pH 7.0. Data refer to several protein concentrations, and temperature after jump is always 25 °C. The continuous line represents a linear least-squares fitting according to the equation:

$$\tau^{-1} = k'([\text{Fe}^{2+}] + [\text{O}_2])_{\text{free}} + k$$

is exothermic [$\Delta H_{\text{ov.}} = -33.1 \pm 3.3 \text{ kJ/mol}$ ($-7.9 \pm 0.8 \text{ kcal/mol}$) for O₂ in solution] (see Fig. 1 inset).

Kinetics with O₂

O₂-binding kinetics measured by temperature jump indicate the presence of a fast bimolecular relaxation process followed by a slower relaxation ($\tau_{\text{II}} = 30 \text{ ms}$), which seems to be independent of O₂ concentration, and may reflect dimer dissociation, but which has not been investigated further.

Although the relaxation time of the slow phase was one order of magnitude slower than the fast phase under any investigated experimental condition, its contribution was always subtracted in the calculation of the fast relaxation time, which always represents the predominant part of the whole relaxation amplitude (approx. 70%). The resulting reciprocal relaxation time for the fast bimolecular process at several reactant concentrations yields an association rate constant $k' = 3.3 \times 10^7 \pm 0.15 \times 10^7 \text{ M}^{-1} \cdot \text{s}^{-1}$ (Fig. 2). Because of the large uncertainty associated with the determination of the dissociation constant from the plot in Fig. 2 ($k = 31 \pm 35 \text{ s}^{-1}$), an independent determination of k was carried out (at different temperatures spanning from 30 to 8 °C) by using the reaction with dithionite in the stopped-flow apparatus. At each temperature the kinetic process is represented by a single exponential ($k = 36 \text{ s}^{-1}$ at 25 °C). The ratio between the association rate constant from temperature jump and the dissociation rate constant from stopped flow (k'/k) agrees remarkably well with the equilibrium constant obtained from the binding isotherm. Activation parameters are given in Table 1.

Kinetics with CO

The CO dissociation time course, similarly to that

Table 1. Transition-state parameters at 25 °C for the reaction of Hb Kempsey with O₂ and CO

	Rate constant	$\Delta G^\ddagger$ (kJ/mol)	$\Delta H^\ddagger$ (kJ/mol)	$\Delta S^\ddagger$ (kJ·mol ⁻¹ ·K ⁻¹)
O ₂				
Dissociation rate constant	36 s ⁻¹	+65.4	+51.0 ± 5.0	-46.4
Association rate constant	3.3 × 10 ⁷ M ⁻¹ ·s ⁻¹	+31.0	+18.0*	-42.5
CO				
Dissociation rate constant	0.017 s ⁻¹	+83.7	+123.8 ± 10.0	+135.1
Association rate constant	5.7 × 10 ⁶ M ⁻¹ ·s ⁻¹	+36.0	+16.3 ± 0.4	-86.1
	4.7 × 10 ⁵ M ⁻¹ ·s ⁻¹	+41.0	+6.3 ± 0.4	-116.3

* Not directly measured, obtained according to the relationship $\Delta H^\ddagger_{\text{on}} = \Delta H^\ddagger_{\text{off}} + \Delta H_{\text{ov.}}$.

Fig. 3. CO binding to Hb Kempsey after flash photolysis

Lower time scale corresponds to the progress curve at [CO] = 500 μM (□); upper time scale relates to the progress curve at [CO] = 20 μM (■). For both progress curves [Hb] = 3.5 μM, and therefore the total amplitude is the same (see the symbol □■). For the curve at [CO] = 500 μM the smaller observed amplitude is due to the fact that a fraction of the reaction takes place within the dead time of the instrument (approx. 200 μs). The continuous lines are manually drawn through the experimental points. Buffer: 0.2 M-Bistris/HCl, pH 7.0; temperature: 20 °C.

observed for human Hb A (Sharma *et al.*, 1976), corresponds to a single-exponential behaviour.

CO-binding kinetics are characterized by two processes, both corresponding to bimolecular reactions, with second-order rate constants (at 20 °C) of 5×10^6 M⁻¹·s⁻¹ and 4.4×10^5 M⁻¹·s⁻¹; the same behaviour is observed with the use of either flash photolysis or stopped flow, in contrast with what is normally found with Hb A (Gibson, 1959; Antonini & Brunori, 1971). The ratio between slow phase and fast phase is essentially independent of CO concentration from 20 to 500 μM (see Fig. 3), which excludes the possibility that the kinetic heterogeneity reflects a slow conformational isomerization with a relaxation time in the range 0.3–300 ms (see also Bunn *et al.*, 1974). A very slow quaternary

transition is also unlikely to be responsible for such a kinetic heterogeneity, as supported by the observation that the slow component predominates when CO is mixed with partially saturated ($\bar{Y} \approx 0.4$) Hb Kempsey, indicating that at equilibrium the fast-reacting component is the one with higher affinity (see Fig. 4). Moreover, additional stopped-flow experiments in which deoxy-Hb Kempsey was mixed with substoichiometric concentrations of CO, under conditions where the percentage of dimers is below 20% (Pettigrew *et al.*, 1982), show a decrease in the amount of the slow kinetic component, which disappears when the CO/haem ratio is $\leq 0.5:1$ (see Fig. 4). These findings suggest that the two kinetic components are not in equilibrium.

The activation parameters of the association and dissociation rate constants for CO are also reported in Table 1.

Fig. 4. CO binding to Hb Kempsey in stopped flow at different CO concentrations and initial saturation degree ($\bar{Y} = \bar{Y}_{t=0}$)

Buffer: 0.2 M-Bistris/HCl, pH 7.0. All concentrations refer to after mixing. [Hb] = 10 μM (haem). ○, $\bar{Y} = 0$, [CO] = 10 μM; △, $\bar{Y} = 0$, [CO] = 3 μM; ■, $\bar{Y} = 0.4$, [CO] = 10 μM. Temperature: 20 °C.

DISCUSSION

Previous work on Hb Kempsey (Bunn *et al.*, 1974) indicated a strong displacement of the allosteric equilibrium towards the R-state, even in the deoxygenated derivative, and this first-order interpretation is substantially confirmed in the present work. The new data, together with those already published (Bunn *et al.*, 1974; Matsukawa *et al.*, 1981), show that the kinetic and thermodynamic parameters in the absence of phosphates have values close to those reported for R-state of human Hb A and different from those of the T-state. Thus ligand binding in Hb Kempsey occurs essentially within the R quaternary state.

Table 1 offers a complete picture of the kinetic parameters that characterize the O₂ and CO binding to Hb Kempsey.

CO dissociation kinetics are characterized by a high activation enthalpy, similarly to Hb A (Antonini & Brunori, 1971), confirming that this rate is purely entropy-driven. Conversely, in the case of O₂ dissociation the formation of the transition state brings about an entropy decrease.

In the ligand-binding process a more negative activation entropy is observed for CO; therefore, in spite of the larger activation enthalpy, the faster association rate observed for O₂ can be attributed uniquely to an entropically more favourable transition state. This appears especially evident for the slower kinetic component of CO binding, which is also characterized by the lowest activation enthalpy.

N.m.r. spectra of stripped deoxy-Hb Kempsey, probing the N_δ atom of the proximal imidazole (Nagai *et al.*, 1982), display two distinct resonances of similar amplitude, attributed by those authors to different conformational states of the proximal site; from the resonance shifts, the local conformation of the two forms appears very similar to those characteristic of the T and R structures of Hb A. Since the two species probed by n.m.r. are in slow equilibrium, if at all, Nagai *et al.* (1982) suggest that the protein, in the absence of O₂, exists partially in a T-like state, casting some doubt on the use of deoxy-Hb Kempsey as a model for the R-state of tetrameric haemoglobin. We propose a correlation between the heterogeneity detected by n.m.r. and that involved in the CO-binding kinetic behaviour; if this is so the kinetics of CO binding [and notably the independence of the fraction of the slow phase on CO concentration and the kinetics of ligand binding to partially liganded Hb Kempsey (see the Results section and Fig. 4)] appear to exclude a slow quaternary transition of the unliganded tetramer. Moreover, if the percentage of dimers is calculated (Pettigrew *et al.*, 1982), the contribution of the slow phase to the binding of CO in tetrameric Hb Kempsey is 50 ± 5% at both protein concentrations investigated (i.e. 3.5 μM- and 20 μM-haem).

On the basis of these considerations, we propose that the kinetic heterogeneity may be attributed to a functional inequivalence in the CO-binding between the two types of polypeptides within an R-state tetrameric haemoglobin. This conclusion, which appears the only one capable of fully explaining the kinetic behaviour reported in Fig. 4, appears quite novel, since no significant subunit heterogeneity has been reported previously for the R-state of haemoglobin. According to

this interpretation, the two n.m.r. resonances probing the proximal site (Nagai *et al.*, 1982) would be attributed to different tertiary conformations in the two types of subunits within the R quaternary state of the macromolecule. Thus assembly of deoxy dimers to deoxy tetramers brings about some peculiar subunit interactions that alter the proximal site of only one type of subunit.

The presence of a single bimolecular process observed for O₂ binding to Hb Kempsey indicates that the effects reported for CO do not appear to influence O₂ combination. This seems very reasonable on the basis of previous data on the kinetics of O₂ binding to Hb A (Ilgenfritz & Schuster, 1974; Sawicki & Gibson, 1977a,b) as compared with CO binding (Sawicki & Gibson, 1976), since it is unanimously recognized that the quaternary structural change brings about a marked change in the association rate constant only for CO. Moreover, it has been demonstrated that the interaction between the haem iron and the proximal histidine residue plays an important role in controlling the reactivity of haemoproteins towards CO (Coletta *et al.*, 1985).

The behaviour of Hb Kempsey seems to mirror the mechanism proposed for CO binding to Hb Zürich [His-E7(63)β → Arg] (Giacometti *et al.*, 1977). Thus in the case of Hb Zürich analysis of the kinetics of CO binding led Giacometti *et al.* (1977) to propose that, although the quaternary state of the deoxygenated Hb corresponds to T₀, the combination rate constant for CO of the abnormal β-chains is indeed similar to that of the R-state. This kinetic heterogeneity within one quaternary state is lost (or much smaller) in the binding of O₂ (Giacometti *et al.*, 1980). In the case of Hb Kempsey the opposite situation seems to prevail.

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