Kinetic Activation Volumes of the Binding of Oxygen and Carbon Monoxide to Hemoglobin and Myoglobin Studied on a High-Pressure Laser Flash Photolysis Apparatus†

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ABSTRACT: The kinetics of the reaction of oxygen and carbon monoxide with hemoglobin (Hb) and myoglobin (Mb) were studied as a function of pressure up to 2760 bar by means of a ruby laser flash photolysis apparatus. The kinetic volumes of activation (ΔV^*) for the binding of O_2 were both positive (Mb, 8; Hb, 5 cm^3 mol^{-1}) and for CO both were negative (Mb, −9; Hb "fast" and "slow" reactions, −3 and −21 cm^3 mol^{-1}), indicating that the interactions of these two ligands may be different in the transition state. The differing values of ΔV^* for the "fast" and "slow" reactions of Hb with CO indicate that different structural changes occur upon binding. The values of ΔV^* have been rationalized in terms of a stereochemical mechanism for O_2 binding and compared with estimates based on the contributions to ΔV^* that would be expected from consideration of simple model compounds.

The reactions of hemoglobin and myoglobin with CO and O_2 have been the object of much study. A summary of much of the kinetic and equilibrium work of reactions of hemoglobin and myoglobin has been given by Antonini and Brunori (1971). Though many of the general features of the kinetics of binding of CO and O_2 to Hb[1] are known, the detailed step-by-step mechanism is not. Perutz (1970) has proposed a stereochemical mechanism in which the binding of O_2 to Hb is accomplished by structural changes in the subunits, triggered by shifts of the iron atoms in the porphyrin and by steric effects of oxygen on the subunit. It is the purpose of the present study to determine volumes of activation, ΔV^*, for the binding of CO and O_2 to Hb and Mb by measuring the influence of pressure on the rate constants for binding, and to rationalize the results in terms of current theories regarding these reactions. Changes in both the tertiary and quaternary structures have been proposed for the binding of CO and O_2 and ΔV^* may provide a useful probe for conformational changes.

Kauzmann (1959) and Rasper and Kauzmann (1962) have pointed out that the partial molal volume of a protein in solution can be considered to be made up of three increments: (i) a constitutive volume, determined by the van der Waals radii of all of the atoms; (ii) a conformational volume, determined by voids and compressed regions of the polypeptide chain which fail to pack perfectly with one another, and (iii) a solvation volume, due to the way in which the protein alters the solvent within its sphere of influence. The ΔV^* for reactions of proteins may be affected by changes in some or all of these increments in addition to bond making or breaking processes.

The theoretical basis for the influence of pressure on protein reactions has been given by Laidler (1958) and Johnson and Eyring (1970). The influence of pressure on the kinetics of the reaction of small molecules, mainly organic, has been recently reviewed by several authors (Whalley, 1964; Le Noble, 1965; Kohnstam, 1970). Much of the earth's surface is covered with water to depths that result in pressures of hundreds of bars, so that the influence of such pressures on biological processes is of importance in understanding organisms that inhabit these regions.

In previous papers we examined the influence of pressure on some simple ligand substitution reactions of aquo Ni^{2+} and Co^{2+} (Caldin *et al.*, 1971, 1972) as well as examining CO substitution on heme itself (E. F. Caldin and B. B. Hasinoff, in preparation). An account has been given concerning the high-pressure laser flash photolysis apparatus that was constructed (Caldin *et al.*, 1973); this account also contains a preliminary report on the reaction of Mb and CO. In the present paper we report the results of an investigation of the effects of pressure on the kinetics of the reactions of Hb and Mb with both CO and O_2.

Various lasers have been previously used to dissociate CO and O_2 from Mb and Hb. Schmelzer *et al.* (1972) used a 20-nsec, 694-nm ruby laser pulse to photodissociate HbCO; McCray (1972) used a 20-nsec, 530-nm Nd laser and a 1 μsec 580 nm Rhodamine 6-G-ethanol liquid dye laser to photodissociate both HbO_2 and MbO_2; and Alpert *et al.* (1972) used a 50-nsec, 530-nm Nd laser to photodissociate HbCO and HbO_2.

Experimental Section

Materials. Hemoglobin (bovine, twice crystallized, Koch-Light) and myoglobin (sperm whale, crystallized, lyophilized, Koch-Light) were dissolved in 0.1 M KH_2PO_4-NaOH (pH 7.00) buffer; the solution was filtered through a Millipore filter to remove particulate matter. Concentrations of the heme protein solutions were determined spectrophotometrically using previously published molar absorptivities (Antonini and Brunori, 1971). Hb and Mb were reduced to their ferrous forms with sodium dithionite. Solutions on which CO kinetics were studied contained 0.01% by weight dithionite. Those on which O_2 kinetics were studied contained only equivalent amounts of dithionite added under strict spectrophotometric control to en-

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[1] Abbreviations used are: Hb, HbCO, and HbO_2, ferrous hemoglobin and its carbon monoxide and oxygen complexes without regard to any particular state or degree of binding; Mb, MbCO, and MbO_2, ferrous myoglobin and its carbon monoxide and oxygen complexes; k_{obsd}, observed first-order rate constant for ligand binding; k_s, second-order rate constant; K, equilibrium binding constant; [ ] and [ ]$_0$, equilibrium and total concentrations.

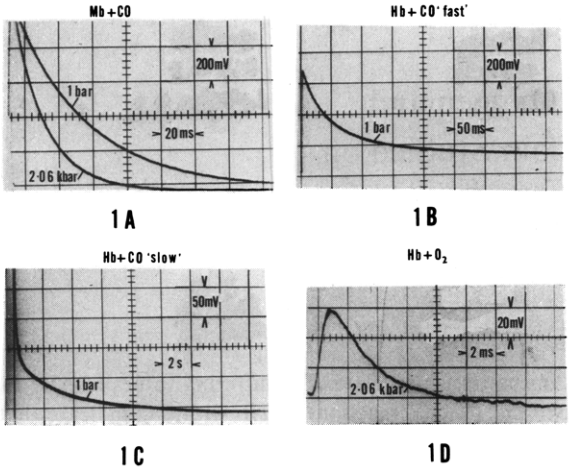

FIGURE 1: (A) Oscilloscope trace of voltage *vs.* time for the recombination of Mb and CO after laser flash photolysis at 48.2° in pH 7.0, 0.1 M phosphate buffer. The $[Mb]_0 = 8.0 \times 10^{-6}$ M and $[CO]_0 = 6.0 \times 10^{-5}$ M. The vertical scale is 200 mV per division and the horizontal scale 20 msec per division. The top trace was measured at 1 bar and the bottom trace at 2.06 kbar. The effect of pressure on the rate constant is clearly demonstrated here. (B) The recombination of Hb and CO after laser flash photolysis at 20.0° and 1 bar. In heme equivalents $[Hb]_0 = 5.8 \times 10^{-6}$ M and $[CO]_0 = 1.2 \times 10^{-5}$ M. The vertical scale is 200 mV per division and the horizontal scale 50 msec per division. This is the first "fast" relaxation observed upon partial photodissociation of HbCO. (C) The recombination of Hb and CO after laser flash photolysis at 20.0° and 1 bar. The conditions are the same as Figure 1B except that the horizontal scale is 2 sec per division. This is the second or "slow" relaxation observed upon partial photodissociation of HbCO. The fast initial vertical portion of the trace is the relaxation observed in Figure 1B. The two exponentials are well resolved in time. (D) The reaction of Hb and O_2 at 20.0° and 2.06 kbar in pH 7.0, 0.1 M phosphate buffer. In heme equivalents $[Hb]_0 = 5.7 \times 10^{-6}$ M, $[O_2]_0 = 2.0 \times 10^{-5}$ M, and $[CO]_0 = 2.7 \times 10^{-6}$ M. The vertical scale is 20 mV per division and the horizontal scale 2 msec per division. The reaction was studied by photodissociating HbCO in the presence of O_2 and following the subsequent reaction of Hb and O_2 which is much faster than recombination with CO.

sure 100% reduction with no excess of dithionite, which reacts rapidly in solution with O_2.

The heme protein solutions were deoxygenated before reduction, by gently bubbling pure nitrogen gas for 15 min through the solutions contained in spectrophotometric cells sealed with rubber caps. The concentrations of the stock saturated CO and O_2 solutions, prepared by bubbling the gas through distilled thermostated water for 15 min at a known temperature and barometric pressure, were determined using Henry's law (In-

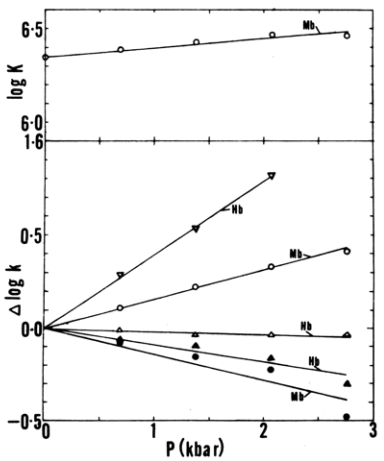

FIGURE 2: Plot of $\Delta \log k$ and $\log K$ *vs.* P. Reaction: (○) Mb + CO, (●) Mb + O_2, (△) Hb + CO ("fast"), (▽) Hb + CO ("slow"), (▲) Hb + O_2. The straight lines are linear least squares calculated.

TABLE I: Temperature Dependence of ΔV^* for the Reaction of Mb and CO.[a]

Temp (°C)	ΔV^* (cm^3 mol^{-1})
10.5	−8.1 ± 0.3
17.8	−8.0 ± 0.2
24.8	−8.9 ± 0.1
37.8	−10.3 ± 0.6
48.2	−10.1 ± 0.3

[a] Rate constants for the determination of ΔV^* from eq 2 were obtained by ruby laser flash photolysis where $[Mb]_0 = 8.0 \times 10^{-6}$ M and $[CO]_0 = 6.0 \times 10^{-5}$ M. The solution was 0.1 M in pH 7.00 phosphate buffer at 1 bar and 25.0°. Standard deviations were calculated from a linear least-squares computer program. Least squares of unit weighting also gave $(\partial \Delta V^*/\partial T)_P = -(0.066 \pm 0.015)$ cm^3 mol^{-1} °K^{-1}.

ternational Critical Tables, 1933). The stock gas solutions were added directly to the spectrophotometer cell with microliter syringes so that the heme protein solution was diluted by only a small known amount.

The water used in making all solutions was triply distilled. Concentrations used to calculate second-order rate constants were on a molality scale reduced to molarity at 1 bar; no correction was made for the compression of the solvent (Le Noble, 1965).

Methods. The high-pressure laser flash photolysis apparatus has been described (Caldin *et al.*, 1972, 1973; E. F. Caldin and B. B. Hasinoff, in preparation). The reaction solution was contained in a Pyrex spectrophotometer cell (0.5 cm^3 volume) sealed by a KEL-F piston and rubber O-rings. The reaction cell was filled and sealed under a nitrogen atmosphere. The recombination of heme protein and ligand was usually followed in the region of the Soret band except for some experiments at high HbCO concentration, where the reaction was followed at 530 nm. Monochromatic light was provided by a continuous spectrum interference filter, half-bandwidth 12 nm. The pulsed ruby laser emits light at 694 nm with an energy density of 9.6 J cm^{-2}, and pulses were 90% complete in 500 μsec.

Small changes in light transmission (<5%) of the reaction solution obtained upon flash photolysis were displayed as a voltage output from a photomultiplier tube on a Tektronix 549 storage oscilloscope. The exponential traces were measured from 35-mm film by a trace matching method (Crooks *et al.*, 1970). Values of k_{obsd} were accurate within 2–10% depending on the system being studied. The experimental uncertainty was dependent on the degree of photodissociation and on the separation in time and relative amplitudes of the "slow" and "fast" reactions.

Representative oscilloscope traces for the laser flash photolysis and recombination of Mb and Hb with CO and O_2 are shown in Figure 1 at various pressures. In Figure 1D, the reaction of Hb and O_2, the rise time of the laser pulse is indicated by the fast initial vertical portion of the trace and is quite well resolved in time from the slower chemical relaxation which was among the fastest observed under the experimental conditions used in this study.

The hydrostatic pressure was maintained in the stainless steel pressure vessel by a pneumatically driven hydraulic pump. Pressure was measured by a Bourdon tube type pressure gauge accurate to ±15 bar. The temperature of the reaction cell was controlled to ±0.1°K.

Checks were frequently made to ensure that pressurization

TABLE II: Activation Parameters and Rate Constants for Reactions of Hb, Mb, and Heme.

Reaction	ΔV^* (cm^3 mol^{-1})	ΔV^*_{corr}	k (M^{-1} sec^{-1}) at 1 bar	ΔG^* (kcal mol^{-1})	ΔH^* (kcal (mol^{-1})	ΔS^* [j] (cal °K^{-1} mol^{-1})
Heme + CO	+2.0 ± 0.4		(2.9 ± 0.3) × 10^7 [a]	7.1	7.5	1.2
Mb + CO	−8.9 ± 0.1		(3.8 ± 0.2) × 10^5 [b]	9.8	4.1	−19.4
Hb + CO (fast)	−0.9 ± 0.3	−3.1	(2.8 ± 0.7) × 10^6 [c]	8.5	5.6[g]	−9.9
Hb + CO (slow)	−22.1 ± 0.5	−21.2	(6.0 ± 3.7) × 10^4 [d]	10.7	9.9[h]	−2.9
Mb + O_2	+7.8 ± 1.3		(1.3 ± 0.3) × 10^7 [e]	7.7	5.5[i]	−7.4
Hb + O_2	+5.2 ± 0.8		(3.2 ± 1.8) × 10^7 [f]	7.2		

[a] At 20.0° in 80% ethylene glycol–water (Caldin and Hasinoff, in preparation). [b] At 25.0°. Compares to k of 5 × 10^5 M^{-1} sec^{-1} (Brunori *et al.*, 1969) and 5.4 × 10^5 M^{-1} sec^{-1} (La Gow and Parkhurst, 1972). [c] At 20.0°. Compares to k of 2.0 × 10^6 M^{-1} sec^{-1} (Schmelzer *et al.*, 1972) and 4.0 × 10^6 M^{-1} sec^{-1} (Antonini *et al.*, 1972) for human Hb. [d] At 20.0°. Compares to k of 9.4 × 10^4 M^{-1} sec^{-1} (Schmelzer *et al.*, 1972), 2 × 10^5 M^{-1} sec^{-1} (Antonini *et al.*, 1972), and 1.1 × 10^5 M^{-1} sec^{-1} (Antonini and Gibson, 1960) by flow. All for human Hb. [e] At 25.0°. Compares to k of 1.5 × 10^7 M^{-1} sec^{-1} (La Gow and Parkhurst, 1972) and 1.9 × 10^7 M^{-1} sec^{-1} (Brunori and Schuster, 1969) by T-jump at 20° and 2.1 × 10^7 M^{-1} sec^{-1} (McCray, 1972) by laser flash photolysis at 23°. [f] At 20.0°. Compares to k of 2 × 10^7 M^{-1} sec^{-1} (Gibson, 1959a), and 4.8 × 10^7 M^{-1} sec^{-1} (Brunori and Antonini, 1972) by T-jump and also to 5 × 10^7 M^{-1} sec^{-1} (McCray, 1972) by laser flash photolysis at 23°. [g] At pH 10.6 (Gibson, 1959b). [h] Overall combination by flow (Gibson, 1959a). [i] Wittenberg *et al.* (1965) for horse Mb. [j] ΔS^* calculated from: $\Delta S^* = (\Delta H^* - \Delta G^*)/T$.

was a reversible process. On many occasions rate constants for recombination of the ligand after photodissociation were measured at 1 bar before and after pressurization, and no change was observed. The small changes in absorbance observed upon pressurization, due to the compression of the solvent and slight shifts in peak positions (Fabry and Hunt, 1968; Zipp *et al.*, 1972), were likewise reversible; so also were the quantum yields at 1 bar. Fabry and Hunt (1968) measured small changes in spectra in the Soret region of a number of Hb derivatives at pressures up to 3 kbar and found these to be reversible. Bridgman and Conant (1929) have indicated that denaturation of HbCO does not occur until 9 kbar, which is a much higher pressure than the maximum used in this study (2.76 kbar). Similarly, Zipp *et al.* (1972) have found that ferric MbF is stable to denaturation at least up to 6.5 kbar.

Results

Influence of Pressure on the Kinetics and Equilibria of the Reactions of Mb with CO and O_2. The recombination of Mb with CO and O_2 upon flash photolysis has been well studied (Antonini and Brunori, 1971), and it has been shown that the rate constant observed for recombination is the same whether determined by rapid flow or by flash photolysis methods. Besides confirming this observation La Gow and Parkhurst (1972) have shown that the eight electrophoretic components of sperm whale myoglobin bind CO and O_2 at the same rate.

Upon flash photolysis of either MbCO or MbO_2 with the ligand at a higher concentration (>5×) than [Mb] ([Mb]$_0$ = 8 × 10^{-6} M) recombination was observed to be first order. For the reaction of Mb with CO, second-order rate constants were calculated from

$$k = k_{obsd}/[CO] \quad (1)$$

This was possible because the rate constant for the dissociation of MbCO is much smaller (0.015 sec^{-1}) than k_{obsd} (3–32 sec^{-1}). For the reaction of Mb with O_2 this is not the case and the expression for k is

$$k = k_{obsd}/([Mb] + [O_2] + 1/K)$$

which is, of course, only valid for small displacements from the equilibrium concentrations. In practice, where the denominator varied from 9 to 48 × 10^{-6} M, k_{obsd} was generally between 10 and 50 times larger than the dissociation rate of MbO_2 (10 sec^{-1}), so that the difference from eq 1 is very small.

From transition state theory (Le Noble, 1965), the activation volume is given by

$$\Delta V^* = -2.303\ RT\ (\partial \log k/\partial P)_T \quad (2)$$

The variation of $\Delta \log k$ ($\log k - \log k$ calculated by linear least-squares analysis at 1 bar) with P is plotted in Figure 2 for the reaction of Mb with CO and O_2. Values of ΔV^* obtained from eq 2 and the slopes of the plots are listed in Tables I and II. Also included in Table II for comparison are previous determinations of the rate constants of these reactions.

Generally it is found that a plot of $\log k$ *vs.* P is curved, indicating that ΔV^* changes with pressure (Le Noble, 1965). Usually the equation $\log k = a + bP + cP^2$ is used. Where there is little or no curvature the equation $\log k = a + bP$ is used. For all the results obtained in this study, both these equations gave an equally good fit to the data as measured by the standard deviation of the fit. Values of ΔV^* reported are those obtained from the equation linear in P. All results quoted with standard deviations were calculated by a weighted linear least-squares computer program.

The reaction of Mb and CO was studied as a function of temperature as well as pressure and the activation parameters obtained are listed in Table III. Values of ΔH^* and ΔS^* were

TABLE III: Pressure Dependence at ΔH^* and ΔS^* for the Reaction of Mb and CO.[a]

P (kbar)	ΔH^* (kcal mol^{-1})	ΔS^* (cal mol^{-1} °K^{-1})
0.001	4.1 ± 0.2	−19.4 ± 0.5
0.69	4.3 ± 0.3	−18.6 ± 1.0
1.38	4.6 ± 0.1	−17.0 ± 0.5
2.06	4.6 ± 0.2	−16.3 ± 0.5

[a] Reaction conditions as in Table I. Standard deviations were calculated from a weighted linear least-squares computer program. Values of ΔH^* and ΔS^* calculated from Arrhenius plots as indicated in results. Least-squares analysis with unit weighting also gave: $(\partial \Delta H^*/\partial P)_T$ = (0.26 ± 0.06) kcal mol^{-1} kbar^{-1} and $(\partial \Delta S^*/\partial P)_T$ = (1.6 ± 0.2) cal °K^{-1} mol^{-1} kbar^{-1}.

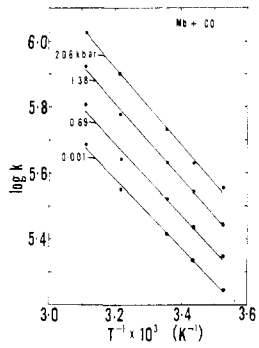

FIGURE 3: Plots of log k *vs.* T^{-1} for the reaction of Mb + CO. The straight lines are linear least squares calculated. From the lower line going up the pressures at which these data were obtained are 0.001, 0.69, 1.38, and 2.06 kbar, respectively.

calculated from the Arrhenius activation energy (E_a) and frequency factor (A) using the relationships $E_a = \Delta H^* + RT$ and $A = (ekT/h)\exp(\Delta S^*/R)$. Values of E_a and A were obtained from the slopes and intercepts of plots of log k *vs.* $1/T$ shown in Figure 3.

It can be seen from an inspection of Table I that ΔV^* varies slightly with temperature by an amount that is larger than the standard deviation.

From a linear least-squares analysis of the ΔV^* *vs.* T data the temperature coefficient of the activation volume is with its standard deviation: $(\partial \Delta V^*/\partial T)_P = -(0.066 \pm 0.015)$ cm^3 mol^{-1} °K^{-1}. From the data of Table III a linear least-squares analysis gives the pressure coefficients of the activation enthalpy and entropy as $(\partial \Delta H^*/\partial P)_T = (0.26 \pm 0.06)$ kcal mol^{-1} kbar^{-1} = (11.1 ± 2.6) cm^3 mol^{-1} and $(\partial \Delta S^*/\partial P)_T = (1.6 \pm 0.2)$ cal °K^{-1} mol^{-1} kbar^{-1} = (0.067 ± 0.007) cm^3 °K^{-1} mol^{-1}. The cross relations among these quantities (Kohnstam, 1970) predict that

$$(\partial \Delta S^*/\partial P)_T = -(\partial \Delta V^*/\partial T)_P$$

$$(\partial \Delta H^*/\partial P)_T = \Delta V^* - T(\partial \Delta V^*/\partial T)_P \qquad (3)$$

The value of $-(\partial \Delta V^*/\partial T)_P$ of (0.066 ± 0.015) cm^3 mol^{-1} °K^{-1} agrees well with the value of (0.067 ± 0.007) cm^3 °K^{-1} mol^{-1} for $(\partial \Delta S^*/\partial P)_T$. Similarly the value of $(\partial \Delta H^*/\partial P)_T$ predicted by eq 3 at 24.8° is $-(8.9 \pm 0.1) - 298(-0.066 \pm 0.015) = -(8.9 \pm 0.1) + (19.8 \pm 4.4) = (10.9 \pm 4.4)$ cm^3 mol^{-1} which is in good agreement with the directly determined value of $(\partial \Delta H^*/\partial P)_T$ of (11.1 ± 2.6) cm^3 mol^{-1}. Kohnstam (1970) has collected most of the reliable values of the pressure and temperature coefficients of the activation parameters for a number of reactions, mainly organic. As is usual ΔV^* and $(\partial \Delta V^*/\partial T)_P$ have the same sign, suggesting that the initial state is more compressible than the transition state.

The change with pressure of the equilibrium constant for the reaction of Mb and O_2 ($K = [MbO_2]/[Mb][O_2]$) was determined by measuring the absorbances of three Mb solutions, one containing no O_2 so that Mb was the predominate species, one containing sufficient O_2 such that there was a mixture of Mb and MbO_2, and one with a large excess of O_2 so that MbO_2 was the predominate species. From the equation $\Delta V^\circ = -2.303RT(\partial \log K/\partial P)_T$ was calculated a value of $\Delta V^\circ = -2.9 \pm 0.2$ cm^3 mol^{-1}. The data with the best fit linear least-squares line are plotted in Figure 2. The equilibrium constant for the reaction of Mb with CO was too large to be determined by this method.

Influence of Pressure on the Kinetics of the Reaction of Hb with CO and O_2. The partial flash photolysis of HbCO shows that there are two relaxations which differ in k_{obsd} by a factor of about 50 (Antonini *et al.*, 1972; Schmelzer *et al.*, 1972; Gibson, 1959a). The fast relaxation is also observed in rapid mixing experiments under some conditions (Antonini *et al.*, 1968). Values of k_{obsd} for both the slow and fast relaxations were measured as a function of pressure; in these experiments CO was in excess, and $[Hb]_0$ expressed in heme equivalents was varied from 5.8 to 50 × 10^{-6} M. Values of k for each process were calculated from eq 1. Plots of $\Delta \log k$ against P are shown in Figure 2. Values of ΔV^* for the slow and fast reactions were calculated from eq 2 by a weighted least-squares analysis of the log k *vs.* P data listed in Table II.

When pressure is applied to the Hb phosphate buffer solution the pH of the buffer solution changes by a small amount. Since the rate constant for the recombination of Hb and CO is pH dependent (Schmelzer, 1972), the change in the rate constant due to the change of pH occurring when the Hb buffer solution is pressurized must be corrected for. When pressure is applied the pH of the phosphate buffer decreases by about 0.5 pH unit per kbar because of the shift in the equilibrium

$$H_2PO_4^- \rightleftharpoons HPO_4^{2-} + H^+$$

for which $\Delta V^\circ = -28.1$ cm^3 mol^{-1} (Hamann, 1963). In the buffered solution the reaction that is being studied is

$$Hb + CO + \nu HPO_4^{2-} \rightarrow HbCO + \nu H_2PO_4^-$$

The quantity ν is the difference in proton binding between Hb and HbCO at a particular experimental pH (kinetic alkaline Bohr effect) and is given by $\partial \log k/\partial$pH. The difference in ΔV^* is equal to ν times the volume change for ionization of the buffer; hence a corrected value of ΔV^* may be calculated

$$\Delta V^*_{corr} = \Delta V^* + \nu \Delta V^\circ \qquad (4)$$

where $\nu \Delta V^\circ$ is the volume change for

$$\nu H_2PO_4^- \rightleftharpoons \nu HPO_4^{2-} + \nu H^+$$

From the data of Schmelzer *et al.* (1972), $\nu = 0.075$ for the fast reaction in the pH range 6–9. Hence from eq 4 the corrected value of ΔV^* is $-0.9 + (0.075 \times -28.1) = -3.1$ cm^3 mol^{-1}. Similarly the slow Hb and CO reaction has a value of $\nu = -0.032$ over the pH range 6–7; hence the corrected value of ΔV^* is $-22.9 + (-0.032 \times -28.1) = -21.2$ cm^3 mol^{-1}. Values of ΔV^*_{corr} are listed in Table II.

The reaction of Hb with O_2 was studied by flash photolysis of HbCO in the presence of O_2. This was preferred to flash photolysis of HbO_2 because the quantum yield for the dissociation of HbO_2 is low (Gibson, 1959a; 1959b). Since the rate constant for the reaction of Hb with O_2 is large (3.2 × 10^7 M^{-1} sec^{-1}), where $[O_2] \gg [CO]$, the *rate* of recombination of Hb with O_2 will be much faster than that with CO. The second-order rate constants for the reaction of Hb with O_2, calculated from $k = k_{obsd}/[O_2]$, were determined at a series of pressures and are plotted in Figure 2. For these experiments $[Hb]_0$, expressed in heme equivalents, was varied from 4.2 to 5.7 × 10^{-6} M. Values of ΔV^* were obtained from eq 2 and a linear least-squares analysis of the data shown in Figure 2. The results are given in Table II. For this reaction $\nu \sim 0$ (from the data of Gray (1970)), so that no correction to ΔV^* is necessary.

Values of ΔG^* for each of the reactions cited in Table II are plotted against ΔV^* in Figure 4.

The effect of pressure on the equilibrium between human Hb and O_2 was measured by Johnson and Schlegel (1948), who found that at a pressure of 0.69 kbar the equilibrium did not shift when HbO_2 varied from 23 to 95.9% in pH 7.4 phosphate buffer at 36°. Similarly Suzuki *et al.* (1972) measured the effect of pressure on the equilibrium of ethyl isocyanide

with Hb and found an "apparent volume" of -23 cm^3 mol^{-1} for the reaction.

Degree of Photodissociation of MbCO, MbO_2, and HbCO. Quantum yields for the photodissociation of MbCO, MbO_2, and HbCO have been measured (Bücher and Kaspers, 1947; Noble *et al.*, 1967) and tabulated recently (Antonini and Brunori, 1971). Found for the various complexes are the values $\Phi_{MbCO} = 1.0$, $\Phi_{HbCO} = 0.4$, $\Phi_{MbO_2} = 0.03$, and $\Phi_{HbO_2} = 0.008$ (Antonini and Brunori, 1971). When quantum yields obtained upon laser flash photolysis are calculated from the absorbance of the heme protein solution at 694 nm (these values were very low, *i.e.*, $A_{MbCO} = 0.0032$ cm^{-1}, $A_{HbCO} = 0.022$ cm^{-1}, $A_{MbO_2} = 0.00066$ cm^{-1}), the energy output of the laser (9.6 J cm^{-2}), and the observed fractional dissociation of the heme protein complex (for MbCO per cent dissociation = 31%; for HbCO per cent dissociation = 35%; for MbO_2 per cent dissociation = 1.1%), an approximate value of Φ may be calculated. The value is approximate only because of the large degree of dissociation which would cause $A_{694\ nm}$ to change throughout the duration of the laser pulse. Values of Φ calculated in this manner are much smaller than those calculated under static conditions. Found were $\Phi_{MbCO} = 6 \times 10^{-3}$, $\Phi_{HbCO} = 6.2 \times 10^{-3}$, and $\Phi_{MbO_2} = 4 \times 10^{-4}$. The reason for this difference is not known. It is possible that it could be due to the nature of the laser pulse which consists of a train of high power spikes or it may be that the quantum efficiency drops sharply at the higher wavelength (694 nm) used in this study. It should also be noted that this wavelength is, in each case, about 100 nm from the nearest absorption peak.

Pressure was observed to reversibly affect Φ; for MbCO, Φ smoothly decreased by about 8% at 2.76 kbar, for MbO_2 a 30% decrease in Φ was observed. The effect of pressure on Φ_{HbCO} was even more marked, reversibly decreasing sevenfold at 2.76 kbar. It was also noted that the amplitude of the slow Hb–CO reaction was more sensitive to pressure than was the fast reaction; at 1 bar the ratio of the amplitude of the fast reaction to the slow reaction was 4.7 and this smoothly and reversibly increased to 30 at 2.06 kbar. The reasons for these changes in Φ are not known but it has been reported previously (Noble *et al.*, 1967) that Φ is a function of the primary structure of the protein, the solvent composition, and the protein concentration.

Discussion

It has been proposed (Antonini and Brunori, 1971) that the fast change of absorption observed when HbCO is flash photolyzed is due to the Hb dimer present in solution along with Hb tetramer. It has, however, been shown that the fast reaction cannot be due solely to dimeric Hb, because the fraction of the total absorption change attributable to the fast reaction is much larger than the relative concentration of dimeric Hb (Schmelzer *et al.*, 1972; Antonini *et al.*, 1971). Moreover, Amiconi *et al.* (1971) showed that in concentrated solutions of triethylamine hydrochloride, which promotes dissociation of tetrameric Hb, the amplitude of the fast reaction is not correlated with the degree of dissociation; nor was the rate of the reaction with CO significantly changed. The fast reaction is probably due to a species that is partially saturated with ligand in the "oxy" quaternary conformation. It would then follow that the slow reaction between Hb and CO is a reaction with Hb in the "deoxy" quaternary conformation. Moreover, that this is also true for O_2 binding to Hb is evidenced by the fact that the rate constant measured by flash photolysis (3.7×10^7 M^{-1} sec^{-1}) is close to the rate constant of 4.8×10^7 M^{-1} sec^{-1} measured by the temperature jump method (Brunori and Antonini, 1972)

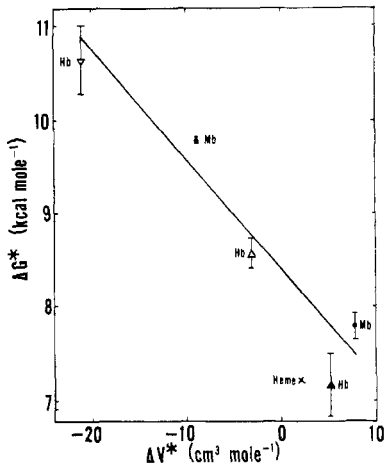

FIGURE 4: Plot of ΔG^* *vs.* ΔV^* for the different reactions. Legend symbols as in Figure 3 except X is heme + CO.

and thus represents reaction of O_2 with Hb in the "oxy" quaternary conformation.

Inspection of the values of ΔV^* in Table II shows a striking difference between the reaction of CO and O_2. The values of ΔV^* for the reactions of O_2 with both Mb and Hb are positive, while those for the reactions of CO are all negative. The difference may be due to a different fit of these ligands in the heme pocket. The interaction between the heme and the protein is very complex (Kendrew, 1962). In Mb there are some 90 contacts between atoms at van der Waals distances; in MbCO there are at least five such contacts between CO itself and protein residues (Antonini and Brunori, 1971) indicating that the heme pocket is quite crowded. Any insertion of a ligand into the heme pocket must result in an alteration of some of these contacts.

It has also been suggested (M. F. Perutz, private communication, 1973) that the difference between CO and O_2 may conceivably arise because histidine E7 is free in HbCO and may form a hydrogen bond to the ligand in HbO_2. The difference would lead to a small overall change in tertiary structure with subsequent volume changes.

The absolute values of ΔV^* obtained are of a similar size measured for some enzyme reactions (Laidler, 1958). It must also be pointed out that they are comparable with the values found for covalent bond formation (~ -10 cm^3 mol^{-1}) or cleavage ($\sim +10$ cm^3 mol^{-1}) in reactions of neutral molecules (Le Noble, 1965). In addition the values of ΔV^* are very small (<0.05%) compared with the total molar volume of either Mb or Hb. This is in contrast to the values of ΔV^* for reactions of small molecules which often represent an appreciable fraction of the molar volume of the reactant molecules.

The simplest model for ligand binding to Mb and Hb is that of two van der Waals spheres coming together; hence ΔV^* would be expected to be about -10 cm^3 mol^{-1} (Le Noble, 1965). This is the simplest mechanism that could be expected as Nobbs *et al.* (1966) has indicated that the sixth coordination position of the Fe atom in Mb does not contain a water molecule. Though bond formation probably contributes to the overall ΔV^*, it cannot be the only contributor given the range of ΔV^* observed ($+7.8$ to -21.2 cm^3 mol^{-1}).

The approximate linear relationship between ΔG^* and ΔV^* shown in Figure 4 indicates that the volumes of activation are related to the work done in forming the transition state. This could indicate that the contribution to ΔG^* is about the same for each of the processes that make up the overall ΔG^*. Hence

processes that decrease the activation volume also decrease the rate constant for ligand binding.

Perutz (1970) has proposed a stereochemical mechanism for the reactions of Hb from X-ray crystal structure determinations. The following discussion attempts to estimate the contribution of various major conformational changes to ΔV^* from experimental values for simple model systems. Though it is recognized that a detailed interpretation of ΔV^* in terms of movements of particular residues in Hb may not be fully warranted it is useful to obtain such estimates of contributions to ΔV^* from various processes in the Perutz mechanism in order to predict relative magnitudes or differences in experimental values of ΔV^*.

The release of Bohr protons (Gray, 1970; Olson and Gibson, 1973) occurs simultaneously with ligand binding; this indicates that conformational changes may take place during the formation of the transition state. From the Perutz mechanism the following processes could conceivably contribute to the observed values of ΔV^*. (i) Bond formation between ligand and Fe should result in a negative volume of activation of about -10 cm^3 mol^{-1} (Le Noble, 1965). (ii) In binding of the first ligand to the α subunit, tyrosine HC2(140) is expelled into the solution from a hydrophobic pocket between helices F and H. This could be expected to lead to a decrease in volume since solvation of hydrophobic compounds is usually accompanied by a decrease in volume (e.g., for benzene, $\Delta V^\circ = -6.2$ cm^3 mol^{-1} (Kauzmann, 1959)). (iii) In the formation of the oxy Hb tertiary structure the amide group linking tyrosine HC2(140) to arginine HC3(141), which is constrained in the deoxy form, becomes capable of rotating freely; it has been estimated that the interchange of a peptide–peptide hydrogen bond for a peptide–water hydrogen bond results in a volume decrease of -2 cm^3 mol^{-1} (Schellmann, 1955). (iv) Also two salt bridges formed by arginine HC3(141) with the opposite α subunit are broken; this will give rise to a volume decrease of -1.6 cm^3 mol^{-1} for each salt bridge (Hamann, 1963; Hemmes, 1972). (v) On the expulsion of tyrosine HC2(140), helix F moves into the center of the molecule, narrowing the pocket between itself and helix H by 1.3 Å; if this movement is not taken up in another part of the Hb molecule a decrease in volume will result. (vi) The α amino groups of valine 1α and the imidazole group of histidine 146β become more accessible in the oxy form, according to Perutz (1970), resulting in the alkaline Bohr effect, the release of protons upon ligand binding. Rasper and Kauzmann (1961) titrated ferric hemoglobin in the pH region where the alkaline Bohr effect occurs and found that the liberation of proton is accompanied by a volume decrease of -2.7 cm^3 mol^{-1}. However, since ν is only 0.075 for the fast Hb reaction the contribution to ΔV^* from this process would be very small and negative. (vii) There may also be a small decrease in volume when the Fe atom becomes "in plane" upon formation of the oxy form. This decrease would be less than -1 cm mol^{-1} on the basis of a simple model (E. F. Caldin and B. B. Hasinoff, in preparation). (viii) The loss of any solvating molecules from the ligand upon entry into the hydrophobic heme pocket would result in a volume increase difficult to estimate but likely to be small.

In addition, reaction of ligand with the α and β subunits differs primarily in two ways. Whereas in the α subunit in the deoxy conformation two salt bridges are broken, only one salt bridge is broken in the β subunit, namely that between histidine HC3(146) and aspartate FG(94); presumably then the volume decrease for this reaction would be about half that for the reaction of ligand in the α subunit in the oxy conformation. Similarly, the contribution to ΔG^* for this reaction would be halved. Secondly, the other process that occurs in the β subunit but not in the α subunit is the formation of space between the porphyrin ring and helix E when the ligand binds to the β subunit; this process would result in a volume increase of an undetermined amount. For both these reasons then, the value of ΔV^* for the reaction of the oxy form should be more positive than that for reaction of the deoxy form. This is in accord with the experimental observation that ΔV^* for CO binding for the fast Hb reaction (deoxy) is more positive than for the slow Hb reaction (oxy) by 18.1 cm^3 mol^{-1}.

To sum up, most of the processes that may appreciably affect ΔV^* (bond formation, solvation of hydrophobic residues, formation of solvent hydrogen bonds, and charge dispersal) will give negative contributions. The large negative volume of activation for the reaction of deoxy Hb (-21.2 cm^3 mol^{-1}) can be understood at least in principle.

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The Acidic Transition of δ-Chymotrypsin†

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ABSTRACT: The behaviors of chymotrypsinogen and δ-chymotrypsin have been studied at acidic pH by optical rotation, absorption, and fluorescence measurements; both proteins show pH-dependent changes. Kinetic experiments using either absorption or fluorescence and a pH jump method have evidenced a slow process which takes place in the enzyme and not in its zymogen; this slow process is controlled by the ionization of a group with a pK of 3, and involves large fluorescence changes. By correcting the changes observed in δ-chymotrypsin at equilibrium from those observed in the zymogen, one may evaluate the variations specifically linked to the ionization of the group of p$K \sim 3$; these variations appear to be very similar to those linked to the ionization of the α-amino group of Ile-16. This result and all the available information make likely the assignment of the pK of 3 to the β-carboxyl group of Asp-194. A more detailed kinetic investigation using fluorescence measurements suggests that δ-chymotrypsin exists under two main conformations, in which this residue has very different pK's. The relevance of the conformational importance of this group in the enzyme to the activation process of the zymogen is also discussed.

One of the critical features of the conformation of chymotrypsin, as revealed by X-ray crystallography, is the existence of an electrostatic interaction between the α-amino group of Ile-16 and the β-carboxyl group of Asp-194; this salt bridge is buried inside the protein molecule and shielded from solvent (Matthews *et al.*, 1967; Sigler *et al.*, 1968). In chymotrypsinogen the α-amino group of Ile-16 forms a peptide bond with Arg-15 and cannot interact with Asp-194. The formation of the Ile-16–Asp-194 interaction appears to be a major event in the activation process, as has been summarized by Hess (1971): "The key which unlocks the inactive conformation is the α-amino group of Ile-16. When this α-amino group is liberated in the conversion of chymotrypsinogen to chymotrypsin at neutral pH, it acquires a positive charge. This positively charged group induces ion pair formation with Asp-194 and the resulting movement of the peptide chains establishes a specific substrate binding site." This remark emphasizes the close connection which exists between the activation process of chymotrypsinogen on one hand, and the role of the Ile-16–Asp-194 interaction in chymotrypsin on the other hand. When lacking the Ile-16–Asp-194 salt bridge (*i.e.*, when the charge of Ile-16 is suppressed by either deprotonation or chemical modification), the enzyme is in a conformation which looks like that of the zymogen, as seen from its circular dichroism spectrum, its optical rotatory dispersion, and its activity toward specific substrates[1] (Oppenheimer *et al.*, 1966; Mc Conn *et al.*, 1969; Ghelis, 1971; Hess *et al.*, 1970; Garel and Labouesse, 1970, 1973; Hess, 1971). We have previously proposed a two-step model for the activation process of chymotrypsinogen: the trypsin catalyzed cleavage of the Arg-15–Ile-16 peptide bond would be an irreversible first step, whereas the second and reversible step

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[1] The activity of the conformation lacking the Ile-16–Asp-194 salt bridge toward nonspecific substrates is still under controversy: we have found it capable of hydrolyzing *p*-nitrophenyl acetate (Ghelis *et al.*, 1970) whereas another report claims it could not (Fersht, 1973). In the case of the related enzyme trypsin, a derivative blocked on its N-terminal α-amino group is still able to hydrolyze *p*-nitrophenyl-*p'*-guanidinobenzoate, a pseudosubstrate, although it is inactive toward specific substrates (Robinson *et al.*, 1973).