form except for the few milliseconds between the photolytic flash and the completion of the reaction. It can be seen that at pH 6 and 7 and at high protein concentrations, the two methods yield similar values for l'. At pH 10.3, however, the flash-photolysis values are all higher than the flow results. This is in accord with our ultracentrifuge results which show that the deoxy form of the protein is more highly aggregated at pH 10.3 than is the ligand-bound form. This preferential dissociation of the ligand-bound form has been observed in other hemoglobins (Andersen *et al.*, 1971; Andersen, 1971).

A further kinetic manifestation of cooperativity in hemoglobins is the difference in l' for the unliganded (Hb) form of the protein and the Hb* ligand-bound conformation, seen on partial photodissociation of HbCO. In *Lumbricus* hemoglobin, Hb* is seen at a much lower fractional photodissociation than in human hemoglobin, implying that the Hb → Hb* transition occurs after a larger fraction of the hemoglobin has bound ligand than in human hemoglobin. A detailed account of these studies will be reported elsewhere (L. Parkhurst and K. Wiechelman, manuscript in preparation).

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Kinetics of Carbon Monoxide and Oxygen Binding for Eight Electrophoretic Components of Sperm-Whale Myoglobin†

Joyce LaGow and Lawrence J. Parkhurst*

ABSTRACT: Sperm-whale myoglobin has been fractionated by isoelectric focusing in Sephadex gels, and oxygen and CO ligand association and dissociation kinetics were measured by stopped-flow and flash photolysis for the eight most abundant fractions. Except for the association rate for CO binding, there appeared to be no significant differences in rates among the various bands for a given reaction. Rates for oxygen dissociation and association determined by replacement reactions were in good agreement with rates determined with dithionite and by flash photolysis, respectively. The rate for CO dissociation determined by NO replacement was homogeneous. Heterogeneous kinetics were observed for the dissociation reaction when $Fe(CN)_6^{3-}$ was used. An evaluation of M, the O_2–CO partition constant, from the kinetic data was in excellent agreement with a direct equilibrium determination.

The electrophoretic heterogeneity of crystalline sperm-whale myoglobin has been known for at least 6 years. As many as five electrophoretic components have been observed on polyacrylamide gel electrophoresis (Hardman *et al.*, 1966) and cellulose acetate (Parkhurst,[1] 1968), while Hapner *et al.* (1968) isolated four components from CM-cellulose columns. When isolated components of many proteins, such as the hemoglobins of trout (Binotti *et al.*, 1971), eel (Yoshioka

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et al., 1968), and lamprey (Rumen and Love, 1963), as well as cytochrome *c* from beef heart (Flatmark, 1967), have been investigated, differences in functional behavior have been found. Previous kinetic work on whale myoglobin has been carried out only on the unfractionated protein. Infrared studies by Caughey *et al.* (1969) suggest the possibility of heterogeneity in the binding of CO by myoglobin. Earlier studies (Gibson, 1959) suggested kinetic anomalies in the oxygen dissociation reaction. For these reasons, it appeared to us of interest to investigate the ligand binding kinetics on electrophoretically pure components of whale myoglobin.

Following the theoretical foundation by Svensson (1961), isoelectric focusing electrophoresis (IEF)[2] has been developed as a powerful analytical technique for detecting protein heterogeneity (Vesterberg and Svensson, 1966; Catsimpoolas, 1970). Numerous workers have detected multiple components in whale myoglobin with this method (Fawcett, 1968; Awdeh *et al.*, 1968; Radola, 1969). IEF has been used in this work on an unusually large preparative scale, allowing the isolation and study of the eight most abundant components of crystalline whale myoglobin. Four reactions, the dissociation and recombination of both oxygen and CO, were studied on all eight bands. Each rate constant was determined in two independent ways for the principal electrophoretic component.

Experimental Section

Compounds. Crystalline sperm-whale myoglobin was purchased from Calbiochem. The carrier ampholines used in isoelectric focusing were obtained from LKB Instruments, Inc., Rockville, Md. Gases, chemically pure, were purchased from Matheson Co., Inc., East Rutherford, N. J. Sephadex was purchased from Pharmacia. Sodium dithionite, Manox Brand, was obtained from Holdman and Harding, Miles Platting, Manchester, England. All other chemicals were reagent grade.

Preparation of Dissolved Gas Solutions. Equilibration of buffers with gases was carried out as described previously (Parkhurst *et al.*, 1970). The concentration of gases was then calculated from the temperature and barometric pressure, using standard solubility tables (Hodgman, 1962).

Fractionation of Myoglobin. Fractionation of the commercial myoglobin was carried out by IEF on layers of Sephadex G-75 gels according to a modification of the method of Radola (1969). Sephadex G-75 (25 g) was swollen in 2 l. of deionized water and allowed to settle. The resulting gel subsequently was washed by decantation and addition of 2 l. of fresh deionized water, the gel being allowed to settle between each washing; this procedure was carried out at least six times. After the last wash, the gel was allowed to settle in a 4-l. beaker for 1–2 hr after which the water was decanted carefully until all but a layer approximately 1 cm above the gel had been removed. This resulted in a ratio of swollen Sephadex to water of approximately 4:1 (v/v). The Sephadex was transferred to a storage bottle and stored at 4°. For a preparative IEF run in which 800 mg of myoglobin was to be fractionated, the Sephadex slurry was cast onto a 20 cm × 20 cm glass plate to which platinum wires that served as electrodes had been cemented 1 cm from the ends of the plate. The gel depth was usually 0.5 cm. The plate was rimmed along its edges on all four sides with modeling clay to keep the Sephadex on the plate. The Sephadex layer was then air-dried until its mass had decreased by 20%. At this point, the Sephadex was firm enough to remove the clay dikes. Myoglobin was dissolved in a minimum amount of deionized water and centrifuged in an International Clinical centrifuge (1800 × *g* for 5 min) to remove insoluble material. The concentration, determined spectrophotometrically on an aliquot converted to cyanomet, was usually 1–1.5 mM on a heme basis. Myoglobin concentrations were based on a value of ϵ_{mM}^{541} 11.3 for cyanomet myoglobin. To this solution was added enough 40% carrier ampholytes of the desired pH range to give a calculated concentration of ampholines 20 times that of the major band, assuming an average molecular weight of 500 for the ampholines (Haglund, 1967). The myoglobin, in 8–10 ml, was applied by means of a Pasteur pipet across the main body of the gel parallel to the electrodes, care being taken to apply the protein no closer than 1 cm away from each electrode. Approximately 0.1 ml of anode and cathode solutions (5% phosphoric acid and 5% ethylenediamine, respectively) were streaked with capillary tubes across the gel surface immediately behind and parallel to the electrodes at the ends of the plate. The sample plate was placed on a Lucite block and covered with another glass plate elevated 5 mm above the gel surface by means of spacers resting on the Lucite base. The entire apparatus was covered with a sheet of Saran wrap to prevent excess evaporation. Power was supplied by means of a Kepko ABC-10, 0–1500-V dc power supply modified for current readout and optional constant current operation; most runs utilized the constant voltage mode. Voltage was applied initially so that the power delivered was no more than 2.5 W. As the run proceeded and the current decreased, the voltage was increased so as to supply approximately 1 W for the duration of the run. All runs were performed at 4°, and were usually completed in 20–24 hr.

Collection and Preparation of the Bands. The focused bands were scraped from the plate. Each gel section was transferred to a 0.9 × 15 cm Sephadex G-25 column. The protein was removed from the ampholines and IEF running gel by eluting with 50 mM potassium phosphate buffer at the pH desired for kinetic measurements. The protein concentrations for all kinetic measurements were between 5 and 10 μM.

pI Measurements. After collection of the gel sections containing the ampholines and the various individual bands into separate test tubes, each tube was capped with a serum stopper and flushed with water-saturated argon for 15 min. The stopper was then removed and pH measurements were made directly on the gel; a stream of argon was directed over the surface of the gel during the measurement. For all pH measurements, an Instrumentation Laboratory pH meter, Model 245 equipped with a combination microelectrode (catalog no. 14043), was employed.

Preparation of Oxymyoglobin. The individual myoglobin components were isolated in the ferric or "met" form, since spectroscopic determination showed that the starting material was largely met. Reduction to the ferrous oxy derivative was accomplished as follows: a tenfold excess of $Na_2S_2O_4$, dissolved in 1 mM deoxygenated NaOH that had been saturated with CO, was added to a deoxygenated, CO-saturated solution of metmyoglobin in 50 mM potassium phosphate buffer, pH 7.0. The resulting CO derivative was passed immediately over a 0.9 × 40 cm Sephadex G-25 column illuminated along its length by a fluorescent lamp; elution, with the phosphate buffer, was complete in about 30 min. The resulting oxymyoglobin was used in kinetic measurements only if the α peak : β-peak absorbance ratio > 1.0 and if there

[2] Abbreviations used are: IEF, isoelectric focusing electrophoresis; MbO_2, oxymyoglobin; Mb, deoxymyoglobin; MbCO, carboxymyoglobin; MbNO, nitric oxide ferromyoglobin.

were no trace of a met peak at 630 nm. Oxymyoglobin prepared in this manner was stable for at least 3 days at 4° before showing any evidence of a met Mb peak at 630 nm.

Kinetic Measurements. Stopped-flow experiments were carried out in an apparatus designed by one of us and described elsewhere (Boelts and Parkhurst, 1971). The stopped flow has a dead time of approximately 1 msec, and 0.1 ml of each reagent is consumed per run. The temperature is read to ±0.004° in the reaction chamber. The flash-photolysis unit, constructed here, has a power output variable up to 200 J and a flash decay half-time of 3 μsec at full power. The sample is contained in a water-jacketed cuvet within a thermostated brass block. The photomultiplier output is fed simultaneously into a Tektronix storage oscilloscope, Model R564B, and a Data General Supernova computer with 4000 words of core memory. The interface unit is similar to that described by Wampler and DeSa (1971). The computer samples the output for 200 time intervals. Absorbance changes and rate constants are computed and displayed numerically less than 1 sec after the end of the reaction on another storage oscilloscope. Hard copy is provided *via* the teletype and by microfilming the oscilloscope display.

Oxygen Dissociation (k). The rate constant for oxygen dissociation was measured in two ways. In the first procedure, the oxymyoglobin, in one syringe, was flowed against a 50 mM potassium phosphate buffer, pH 7.0, that was 0.01 M in $Na_2S_2O_4$. The dithionite consumed the oxygen released by the protein, thus eliminating the reverse oxygen-binding reaction. The reaction was studied at 413 nm. The second method utilized a reaction in which CO replaced the oxygen. In this experiment, oxymyoglobin was flowed against a series of buffers in which the concentration ratio (O_2/CO) was varied, although each experiment was carried out so that (O_2/CO) was kept constant to within 4% during the course of the reaction. The wavelength for observing this reaction was 422 nm.

O_2 "On" (k'). The combination of oxygen was measured in a flash-photolysis experiment. Oxymyoglobin was prepared as described, and then diluted into air-equilibrated buffer containing CO. The CO concentration was one-fifth that of oxygen. The protein was converted to the CO form upon dilution into the CO- and O_2-containing buffer. The CO was photodissociated and the combination of the O_2 with the heme was observed. Under these conditions, the O_2 reaction was *ca.* 150 times faster than the CO reaction. This reaction was studied at 430 nm. A rate for O_2 combination was also obtained from the O_2 replacement studies.

CO "On" (l'). The rate constant for CO "on" was measured by both stopped-flow and flash photolysis. In the former experiment, the protein, in oxygen-free buffer (50 mM potassium phosphate, pH 7.0), was reduced from the met to the ferrous form in a syringe by the careful addition of a few crystals of $Na_2S_2O_4$ until the appearance of reduced myoglobin was observed. The ferrous protein was then flowed against oxygen-free buffer of known CO concentration. The flash-photolysis studies utilized the photosensitivity of the CO derivative. Carboxymyoglobin was prepared by diluting the met protein with oxygen-free buffer of known CO concentration. $Na_2S_2O_4$ was carefully added producing carboxymyoglobin. Dissociation of the CO followed the absorption of the photolytic light from the flash unit, and the time course with which CO and myoglobin recombined was followed. The reaction was studied at 422 and 430 nm in both the stopped-flow and flash-photolysis experiments.

CO Dissociation (l). CO dissociation was measured by means of replacement by NO. The carboxymyoglobins were prepared according to the procedure for oxymyoglobin except that the Sephadex column was not illuminated and the protein was allowed to elute rapidly. After collection, the protein solution was saturated with CO to ensure complete conversion to the carboxymyoglobin form. Just before use, excess CO was removed by passing water-saturated argon over the protein solution for 10 min. The protein was then diluted into O_2- and CO-free buffer and flowed against an oxygen-free NO-saturated buffer solution, and the reaction followed at 422 nm.

An attempt was made to measure CO dissociation by flowing carboxymyoglobin against potassium ferricyanide solutions, at ferricyanide concentrations of 1 mM and 100 μM. This reaction was observed at 422 nm.

Partition Constant (M). The partition constant, M, is the ratio of oxygen (K) to CO (L) equilibrium constants, and is the equilibrium constant for the overall replacement reaction

$$MbO_2 + CO \rightleftarrows MbCO + O_2 \quad (1)$$

$$M = \frac{(MbCO)}{(MbO_2)}\frac{(O_2)}{(CO)} = \frac{K}{L} = \frac{(Mb)(O_2)}{(MbO_2)}\frac{(MbCO)}{(Mb)(CO)} = \frac{k/k'}{l/l'} \quad (2)$$

The ratio (MbCO)/(MbO_2) was determined spectrophotometrically throughout the Soret region in a Cary 14 following equilibration of the 5 μM myoglobin with the various ratios of (O_2)/(CO) in a 500-ml tonometer fitted with a 1-cm pathlength cuvet.

Results

Isoelectric Focusing. In some runs, as many as 15 bands could be seen distinctly; only the first eight (numbering is from the cathode), representing the most abundant components, were collected. The unfractionated crystalline material is mainly metmyoglobin and at least 11 of the bands were also in the met form as evidenced from their absorption spectra; among these were the eight components studied. In all runs, band III had a characteristic red color. Spectroscopic analysis revealed that this band contained a small amount of oxymyoglobin which had a pI identical with band III and therefore focused at the same position on the gel. When the unfractionated myoglobin was converted to oxymyoglobin and subjected to IEF, pH measurements on the bands subsequently formed showed that the oxy component of band III was actually the oxy form of band I. Other red-colored bands were detected on some runs. These focused at pI values more acidic than band III and presumably were the oxy forms of other bands. In analytical IEF runs where the amount of crude myoglobin was small, a minor band was observed which focused very near band I to the anode side. In preparative runs, this band could not be separated cleanly from band I, and was not studied.

For the work reported in this paper, a total of 84 IEF gels were run. On eight of these runs, very careful measurements of pI for all bands were made. The values reported in Figure 1 are averages for these eight runs. The standard deviation for the pI values is ±0.05, except for band I, for which 12 gels were measured, and the standard deviation was ±0.03.

Two pH ranges of ampholines were used, 6–8 and 7–9. Since the pI of band I was measured to be 8.11, this meant that the band focused very near the cathode using the pH 6–8 ampholines, and it was feared that contact with the strongly

TABLE I: Whale Myoglobin Rate Constants.[a]

Band	% Abundance	l' (M^{-1} sec^{-1}) ($\times 10^{-5}$)	l (sec^{-1})	k' (M^{-1} sec^{-1}) ($\times 10^{-7}$)	k (sec^{-1})
1	74	5.4	0.015	1.5	10.0
2	1.2	6.3	0.016	1.5	9.9
3	5.6	6.5	0.016	1.5	10.4
4	5.8	6.3	0.018	1.5	9.8
5	2.6	6.4	0.016	1.5	10.5
6	0.5	6.1	0.020	1.5	10.9
7	0.5	6.9	0.016	1.5	9.1
8	0.4	6.8	0.017	1.4	9.1

[a] Protein concentrations were: 10 μM (before mixing) in stopped-flow and 5 μM in flash-photolysis experiments. Reactions were run in 0.05 M potassium phosphate buffer, pH 7.0, 20°. Per cent abundance refers to per cent of a given band found in crude myoglobin. Approximately 10% remained unfocused or focused in other minor bands.

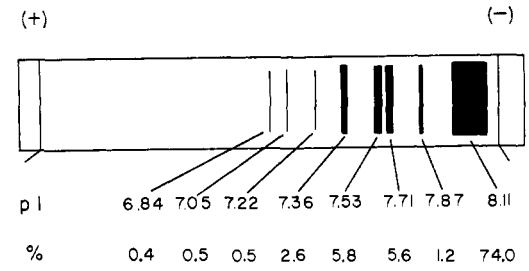

FIGURE 1: Isoelectric focusing pattern of sperm-whale myoglobin on a 5-mm layer of Sephadex G-75 using pH 6–8 ampholines. Per cent refers to the abundance of a given band found in crude myoglobin. Approximately 10% remained unfocused or focused as other, uncollected minor bands.

basic cathode solution might alter the protein. Care was taken to collect only from the middle of the focused band, but several runs were carried out at pH 7–9, where band I would be focused near the middle of the plate, to determine if any differences in pI or kinetic behavior could be detected. No differences in either property were observed.

Individual bands were refocused and no signs of heterogeneity were detected; bands I, II, and V refocused as single bands in individual experiments. Bands III and IV, which focus with less than 1 mm separation, showed slight traces of the other band when refocused separately.

Figure 1 depicts a typical IEF run. Relative abundances based on total myoglobin applied to the gel, as well as pI values, are listed below each band. By weight, approximately one-half of the commercial lyophilized crystalline whale myoglobin is myoglobin (which is 95–98% of the protein present) as determined by conversion to, and spectrophotometric measurement on, the cyanomet derivative. After focusing, the bands were freed from gel and ampholines as described above. Concentrations were determined by conversion to the cyanomet form. Approximately 10% of the crude myoglobin did not focus into bands. This material was located mainly between the first five bands and near the anode. The latter color represented some of the very minor bands, from band IX on, since these were not detectable as discrete bands in every run.

Ampholines have been reported (Vesterberg, 1969) to be removed successfully from proteins by means of Sephadex G-25 columns. We have found that if the protein–ampholine mixture were passed over a column 0.9 × 10 cm, the pH of the resulting protein solution was that of the eluting buffer. If the column were too short, the pH of the eluted protein was intermediate between that of the eluting buffer and of the pI of the protein, suggesting the presence of ampholines. After gel filtration, the myoglobin bands were present in a concentration of 150 μM or more and were subsequently diluted to 5 or 10 μM for the kinetic experiments. Even if the ampholine:myoglobin ratio were 1:1 on a molar basis following gel filtration, 50% binding at 5 μM would imply a $K_{diss} = 2.5 \times 10^{-6}$ M, an affinity ten times greater than that of N_3^- for ferric myoglobin. Ampholines were mixed with desalted bands as well as with the crude unfractionated myoglobin so that the resulting mixture contained an ampholine:protein ratio of 10:1 on a heme basis. The azide association constants were identical for the two ampholine-treated samples (band I and crude myoglobin), the crude myoglobin that had never been in contact with ampholines, and the desalted band I presumably free from ampholines. The CO association rate constant was measured for the same four proteins. In that study, both crude myoglobin samples, treated and untreated, gave identical results, as did the two samples of band I. For reasons given below, the kinetics of band I and crude myoglobin differed slightly for this reaction. We conclude that even if ampholines were present in the sample after gel filtration, it is unlikely that the ligand binding kinetics were perturbed.

Kinetic Measurements. The rate constants measured for the individual bands are summarized in Table I. The letter conventions used for the rate constants are those of Hartridge and Roughton (1923; Roughton, 1934). Values of l' are from stopped-flow measurements. Values of l are from the NO replacement reactions. The values of k' are from flash-photolysis measurements. Values of k are from stopped-flow measurements of the reactions of MbO_2 with dithionite. The standard deviation for a given rate constant on a specific preparation was $<\pm 3\%$. The standard deviation for the average of a rate constant over eight preparations was $\pm 5\%$.

Dissociation of O_2 (k). The reaction of oxymyoglobin with $S_2O_4^{2-}$ can be represented as follows

$$MbO_2 \xrightarrow{S_2O_4^{2-}} Mb + O_2 \quad (3)$$

whereas in the dissociation of oxygen from myoglobin and subsequent replacement by CO, the reaction studied is

$$MbO_2 + CO \underset{k'}{\overset{k}{\rightleftharpoons}} Mb + O_2 + CO \underset{l}{\overset{l'}{\rightleftharpoons}} MbCO + O_2 \quad (4)$$

The rate for a reaction measured by the replacement of one ligand by another, assuming (Mb) is small, and (CO) and (O_2) are constant, is expressed by eq 5.

$$R = \frac{k'(O_2)l + l'(CO)k}{k'(O_2) + l'(CO)} \quad (5)$$

The CO dissociation rate constant, l, is so small that the term in which it appears drops out. Cast in a form suitable

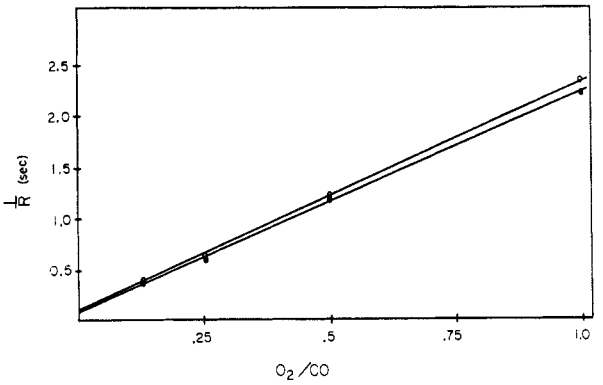

FIGURE 2: Reciprocal of the rate of O_2 replacement by CO *vs.* (O_2/CO) in MbO_2, as measured by stopped flow at pH 7.0, 20°. Buffer composition was 0.05 M potassium phosphate. Myoblogin concentrations (before mixing) were 10 μM. The symbols are: (○) band I; (●) crude myoglobin.

for plotting, the expression simplifies to (Gibson and Roughton, 1955)

$$\frac{1}{R} = \frac{1}{k} + \frac{(k')}{k(l')}\frac{(O_2)}{(CO)} \qquad (6)$$

When the results of the displacement reaction for band I are plotted, the least-squares line in Figure 2 is obtained, and k was determined from the y intercept. The value, 9.2 sec^{-1}, is in very good agreement with the dithionite experiments, in which k was determined to be 10.0 sec^{-1}. The rate constant for the crude material was also determined by both methods; by the dithionite method, k = 10.0 sec^{-1}, while the replacement reaction gave 9.7 sec^{-1}. The rate constant k was also calculated from the slope of this plot and from values for k' and l' obtained from direct kinetic determination. From such a calculation, k = 12 sec^{-1} for band I.

O_2 "On" (k'). Examination of eq 6 shows that the slope, m, for the plot of the replacement reactions is k'/kl'. Thus, k', the on constant for oxygen, can be obtained from $k' = ml'k$, if l' and k are known. If the values 10.0 sec^{-1} and 5.4 × 10^5 M^{-1} sec^{-1} are substituted for k and l', respectively, for band I, and the least-squares slope, 2.2 sec, is used, a value for k' of 119 × 10^5 M^{-1} sec^{-1} is obtained, whereas a direct measurement by flash photolysis gave 148 × 10^5 M^{-1} sec^{-1}.

CO "On" (l'). The binding of CO to the unliganded protein takes place according to the following reaction

$$Mb + CO \underset{l}{\overset{l'}{\rightleftharpoons}} MbCO \qquad (7)$$

The dissociation constant, l, was so small that the reverse reaction could be neglected in our work in which (CO) varied from 50 to 900 μM.

The values of l' obtained from both flash photolysis and stopped-flow experiments agreed within 2%.

The difference in rate constants between band I and the rest of the bands was variable. We have performed over 100 experiments on eight separate electrophoretic preparations by stopped flow on bands I and IV. For seven preparations, we found a difference between the constants amounting to about 16–18%; in the other preparations the variation was within experimental error.

CO Dissociation Rate Constant (l). The CO dissociation rate constant was determined by replacement with NO. The binding of NO takes place so rapidly for hemoglobins and K_{assn} is so high (Gibson and Roughton, 1957) that there is virtually no contribution from the reverse reaction with CO so that the reaction may be represented by the equation

$$MbCO + NO \xrightarrow{l} MbNO + CO \qquad (8)$$

In these experiments, the effect of the reverse reaction was further reduced by removing nonliganded CO from the MbCO solution immediately prior to the kinetic experiments; this was accomplished by flushing with water-saturated argon for 15 min.

In the experiments on band I with ferricyanide, the kinetic traces were markedly biphasic. Values for l of 0.011–0.009 sec^{-1} and 0.06–0.03 sec^{-1} were obtained for the slow (62% of the total reaction) and fast phases when the concentration of $Fe(CN)_6^{3-}$ varied from 100 to 1 μM, respectively. These rate constants are to be compared with the value 0.015 sec^{-1} obtained by the NO replacement method in which the reaction was homogeneous over 4 half-lives.

Partition Constant (M). Two direct determinations of M for band I each gave a value of 22.5 at 20°, pH 7.0. This constant also can be evaluated from the rate constants of Table I according to eq 2. Such an evaluation gives M = 24.3.

Discussion

Edmundson (1965) has attributed the appearance of multiple components in whale myoglobin to successive deaminations in the protein, thus introducing the charge differences reflected in the pI values. It has been suggested (Satterlee and Snyder, 1969) that exposure to electrolysis products during the IEF run caused the reduction of metmyoglobin to the oxy derivative in the case of the bovine myoglobin system they studied. Contamination of electrolysis products does not seem likely in the pH 7–9 runs employed in many preparations, since band I focuses in the middle of the plate, approximately 9 cm from the electrode solution, and, furthermore, the red component of band III is still seen on these runs. When the crude crystalline protein is oxidized either with KNO_2 or $K_3Fe(CN)_6$ prior to IEF, and the oxidant removed by gel filtration, the characteristic red color of band III does not appear, either at pH 7–9 or 6–8. The spectrum of band III after isolation from these experiments is that of metmyoglobin.

The values reported for k, the oxygen dissociation constant, in this paper for band I and crude myoglobin are in excellent agreement with a previously reported value (10 sec^{-1}) for crude whale myoglobin (Gibson, 1959). The agreement for k obtained by the replacement reaction and by the dithionite method is excellent; the rates are identical within experimental error. In the calculation of k from the slope of Figure 2, the error in k must include errors in m, l', and k'. The difficulties experienced by earlier workers may derive from poor oxymyoglobin preparations. The presence of even a small amount of metmyoglobin in the oxymyoglobin sample can affect the rate observed, since the reaction

$$Mb(III) \xrightarrow{S_2O_4^{2-}} Mb(II) \qquad (9)$$

may contribute to the observed reaction. The observed reaction at 430 nm will be heterogeneous and will increase in rate with increasing dithionite concentration if Mb(III) is present. The influence of any possible secondary reaction

(Mb(III) → Mb(II) was eliminated by observing the deoxygenation at 413 nm, the isosbestic wavelength for met and reduced (unliganded) myoglobin.

The value for k', the oxygen combination constant, reported in this paper for band I is 17% higher than that reported by Gibson in 1956 (Gibson, 1956) (125×10^5 M^{-1} sec^{-1}) but is 1.5 times that reported elsewhere (Gibson, 1959) (100×10^5 M^{-1} sec^{-1}). The difference between the two k' values obtained from direct measurement by flash photolysis and from the slope of the plot of the replacement reaction is not exceptional since k' obtained from the slope of the replacement reaction is calculated from three constants determined in independent experiments.

We were unable to find a value reported for l, the CO dissociation constant, for whale myoglobin elsewhere in the literature. While the NO replacement reaction for determination of l is technically more difficult to perform, it appeals to be the method of choice over the ferricyanide reaction, which is more commonly used. Values cited for l for horse myoglobin, 0.04 sec^{-1} (Gibson, 1964), are derived from measurements (Millikan, 1936) using ferricyanide. In view of our results, it might be well to reexamine l for those heme proteins, such as horse and *Aplysia* (Wittenberg *et al.*, 1965) myoglobins, where the reported l was determined by ferricyanide. The value of M calculated from the kinetic constants (24.3) is in excellent agreement with the direct determination (22.5). This agreement suggests a high level of consistency among the rate constants and further points to the value of l from NO replacement as being the correct value.

All reactions for all eight bands appeared identical within experimental error with the exception of the binding of CO, l'. The value reported in this paper for this constant for band I is in good agreement with that reported (5×10^5 M^{-1} sec^{-1}) elsewhere for crude whale myoglobin (Gibson, 1959; Parkhurst and Gibson, 1967). The difference seen in most experiments between band I and the other bands, 16–18%, appears to be significant. This reaction seems to be sensitive to the age of the protein. In the preparation in which the proteins had been fractionated for more than a week, the rates appeared identical within 3%. In all other experiments, band I reacted more slowly than the other bands. This result may be related to the findings of Rudolph *et al.* (1972), in which calorimetric investigations on the unfractionated myoglobin gave evidence for at least two functionally distinct components. The spectroscopic features reported by Caughey *et al.* (1969) may also be related to our observed kinetic heterogeneity. If the charge differences on the proteins cause any changes in conformation that affect the heme site, binding is not noticeably affected except possibly for CO combination.

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