TETRAMER-DIMER DISSOCIATION OF CARBOXYHEMOGLOBIN IN THE ABSENCE OF DITHIONITE

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ABSTRACT The generally accepted value for the tetramer-dimer dissociation constant $K_{4,2}^{\mathrm{L}}$ of carboxyhemoglobin in pH 7.0 phosphate buffer lies in the range 1–2 μM. Previous determinations of this quantity have generally involved addition of dithionite to samples to exclude oxygen. We report flash photolysis experiments on carboxyhemoglobin in the absence of dithionite which suggest that $K_{4,2}^{\mathrm{L}}$ is 0.2 ± 0.05 μM. Addition of dithionite to our samples resulted in an order of magnitude increase in $K_{4,2}^{\mathrm{L}}$ in good agreement with previously published results. The mechanism of this increase in dissociation has not been determined with certainty. However, impurities, possibly metal ions, are required in addition to dithionite to produce this effect. Dithionite did not increase $K_{4,2}^{\mathrm{L}}$ for phosphate buffer solutions treated with Chelex 100 analytical grade chelating resin. Addition of bovine serum albumin to untreated buffer solutions before addition of dithionite was found to prevent increased dissociation. The sulfhydryl-reducing agents dithiothreitol and β-mercaptoethanol were found to protect against the effect of dithionite and to reverse its effect on $K_{4,2}^{\mathrm{L}}$ if they were added after the dithionite. The interaction of the unknown impurities with dithionite to produce increased values of $K_{4,2}^{\mathrm{L}}$ could be mimicked by addition of Cu^{2+} ions in concentrations of <1 μM to buffer treated with Chelex 100 resin.

INTRODUCTION

A number of physical techniques have been applied to the determination of $K_{4,2}^{\mathrm{L}}$, the tetramer-dimer dissociation constant of carboxyhemoglobin. The presently accepted value for $K_{4,2}^{\mathrm{L}}$ in pH 7.0 phosphate buffer lies in the range of 1–2 μM as suggested by ultracentrifugation (1) and flash photolysis experiments (1–3). In these experiments sodium dithionite was used to maintain oxygen-free conditions. Experiments in the presence of dithionite typically show, when repeated, a large scatter in the $K_{4,2}^{\mathrm{L}}$ values obtained. See, for example, reference 3, Fig. 4.

We report flash photolysis experiments on carboxyhemoglobin in the absence of dithionite, which give values for $K_{4,2}^{\mathrm{L}}$ in the range 0.1–0.3 μM. Addition of dithionite to our samples increased the apparent dissociation constant by about an order of magnitude in good agreement with previous experiments.

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MATERIALS AND METHODS

The tetramer-dimer dissociation constant is given by Eq. 1.

$$K_{4,2}^{\mathrm{L}} = \frac{D^2}{T}, \tag{1}$$

where D and T are the molar concentrations of dimeric and tetrameric species. We have used the fraction of rapidly rebinding hemoglobin observed following full photolysis of dilute carboxyhemoglobin solutions to calculate the tetramer-dimer dissociation constant, according to Eq. 2 taken from reference 4.

$$K_{4,2}^{\mathrm{L}} = [\mathrm{HbCO}]\,[\alpha^2/(1-\alpha)] \tag{2}$$

where [HbCO] is the total carboxyhemoglobin concentration on a heme basis and α is the fraction of fast reacting material observed at 436 nm following photolysis.

Calculation of $K_{4,2}^{\mathrm{L}}$ in this way has been used throughout as a matter of convenience because it allows a large number of individual measurements to be summarized by a single number. Detailed examination of the concentration dependence of the reaction showed that the apparent value of $K_{4,2}^{\mathrm{L}}$ was larger at low hemoglobin concentrations in the presence of dithionite. Such an effect suggests that, at least in part, the effect of adding dithionite is to bring about the formation of a definite amount of rapidly reacting material which can be seen by flash photolysis. This idea is consistent with the hypothesis put forward later in this paper that another impurity acts in concert with dithionite to produce increased dissociation.

The conventional flash photolysis apparatus used has been described previously (5). Solutions of human hemoglobin were prepared as detailed (6) and used within 1 wk. All experiments were carried out at 20°C in 1-cm path length serum stopper-sealed cells. After the sealed cells were flushed with nitrogen gas, they were rinsed and filled with nitrogen bubbled buffer by injection through the stoppers (7). Experimental samples were prepared by injecting deoxyhemoglobin, carbon monoxide saturated buffer, and other reagents directly into these cells with gas-tight microliter syringes. Preparation of the samples in cells allowed the effect of changes in one component of the mixture to be easily examined. For example, the hemoglobin concentration dependence could be studied by injecting successive portions of deoxyhemoglobin into the known volume of solution in the cell. By performing experiments with hemoglobin freed from residual CO (7) the extent of oxygen contamination of the samples could be measured using laser photolysis (7) and was found to be <0.3% saturation.

Reagent grade chemicals were used in all cases. Chelex 100 analytical grade chelating resin (Bio-Rad Laboratories, Richmond, Calif.) was prepared and used to treat some samples of phosphate buffer in the manner described by Willard et al. (8). The sodium dithionite used was Manox brand obtained from Hardman and Holden, Miles Platting, Manchester, England.

Data were collected using two different methods. First voltage changes observed after photolysis were digitized at two sampling rates and stored in the memory of a PDP 8A computer (9). After conversion to absorbance changes the fraction of fast (dimer) rebinding was determined by fitting the kinetics to two exponentials (2). In all cases the CO concentration was at least five times the hemoglobin concentration (on a heme basis) to insure that the reaction could be adequately described by two pseudo first-order processes. Under the conditions of low CO concentration employed in these experiments the rate of the quaternary conformational change (10) is very much larger than the fast rebinding rate of the quickly reacting deoxyhemoglobin present immediately after the flash. Thus the fast rebinding observed after photolysis was not due to deoxyhemoglobin in the high affinity conformational state.

The second method for data collection involved measuring the total voltage excursion following full photolysis, using a storage oscilloscope (Tektronix 5103N, Tektronix Inc., Beaverton, Ore.), and then measuring the slow voltage excursion directly from the oscilloscope display using a straight edge to project the change back to the time of the firing of the flash. In this case the slow change is displayed using an oscilloscope time-base scale so that the total time range displayed across the screen is about half of the half time for the slow rebinding. The success of this simple method for separating the two phases depends upon the 30-fold difference in the CO rebinding rate of deoxyhemoglobin and dimers (2).

Both methods gave the same results for $K_{4,2}^{L}$ within experimental error. Low hemoglobin concentrations were used in these experiments so that comparable contributions were observed from the fast and slow phases of CO rebinding. Under these conditions errors in the calculation of $K_{4,2}^{L}$ are minimized.

RESULTS AND DISCUSSION

Most of the experiments reported in this work have been performed in 0.1 M pH 7.0 phosphate buffer, since the tetramer-dimer dissociation of carboxyhemoglobin has been most thoroughly studied under these conditions (1–3). In addition, parallel ultracentrifugation and flash photolysis experiments (1) performed in this buffer resulted in values for $K_{4,2}^{L}$ which agreed within experimental error. All of these earlier experiments were performed in the presence of dithionite to eliminate problems associated with oxygen contamination of samples.

Fig. 1 gives the relation between hemoglobin concentration and the fraction of rapidly recombining hemoglobin observed in the absence of dithionite for hemoglobin prepared from three blood samples. These data represent the largest sample to sample variation obtained in the absence of dithionite. Assuming that the rapid part of the reaction is due to hemoglobin dimerization gives a reasonable description of the data (solid curves). As seen in Fig. 1 there is sample to sample variation in $K_{4,2}^{L}$ which appears to be beyond experimental error. We were unable to determine the origin of this variation. The value suggested for $K_{4,2}^{L}$ by the data presented in Fig. 1 is nearly an order of magnitude lower than values found previously in the presence of dithionite (1–3). We have neglected association of deoxy dimers following photolysis because the reported rate constant for this process is ~0.5 $\mu M^{-1}s^{-1}$ (11,12) and in our experiments the rate of CO binding to dimers was 100 or more times grater than the expected rate of dimer recombination.

Table I gives average values for the apparent dissociation constant $K_{4,2}^{L}$ calculated from the fraction of rapidly reacting hemoglobin observed following photolysis of carboxyhemoglobin. The errors represent 1 SD for each reported value. In most cases, addition of dithionite caused

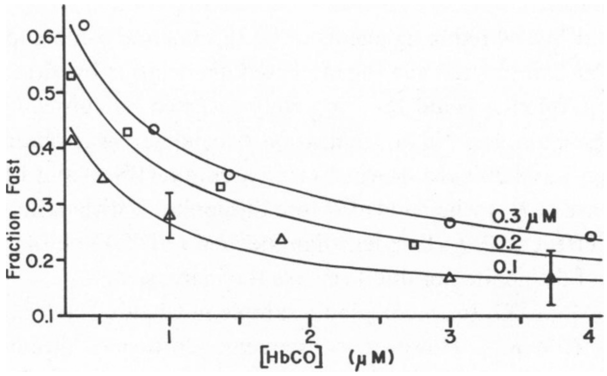

FIGURE 1 Fraction of hemoglobin reacting rapidly with CO in the absence of dithionite after photolysis of HbCO in 0.1 M phosphate buffer at 20°C with a total CO concentration (free and bound) of 25 μM. The solid curves were calculated from Eq. 2. The value taken for $K_{4,2}^{L}$ is given above each curve.

TABLE I
APPARENT TETRAMER-DIMER DISSOCIATION CONSTANTS

Solution conditions	$K^{L}_{4,2}$	
	no dithionite	with dithionite
	μM	μM
Kpi 0.1 M	0.2 ± 0.05	2 ± 1
Kpi 0.01 M	0.15 ± 0.05	0.3 ± 0.1
bis-Tris 0.1 M +500 mM DPG +0.1 M NaCl	0.4 ± 0.2	1.4 ± 0.7
Kpi 0.1 M +1 M NaCl	18 ± 4	18 ± 4
Kpi 0.1 M (Chelex-100 treated)	0.2 ± 0.05	0.3 ± 0.1

All experiments were performed at 20 ± 1°C and pH 7.0.

a significant increase in the apparent value of $K^{L}_{4,2}$. No dependence on dithionite concentration was observed between 5 and 500 μM. The value 2 ± 1 μM obtained for 0.1 M phosphate buffer with added dithionite agrees well with values reported in the literature (1–3). The value 1.4 ± 0.7 μM found for bis-Tris buffer with dithionite is the same as that reported by Gray (2). Addition of dithionite had no significant effect on the dissociation of hemoglobin in the presence of 1 M NaCl. Our value for $K^{L}_{4,2}$ (18 μM) agrees well with the value 20 μM found by Guidotti (13) in osmotic pressure measurements.

Changes in the concentration of the phosphate buffer were found to have a dramatic influence upon the effect of dithionite addition to hemoglobin samples. As seen in Table I, addition of dithionite to hemoglobin samples in 0.01 M phosphate buffer caused only a small increase in $K^{L}_{4,2}$. This could be explained by the presence in the buffer of other impurities, which together with dithionite cause the increase seen in $K^{L}_{4,2}$. To test this hypothesis some pH 7.0 phosphate buffer solution was treated with Chelex 100 resin as described by Willard et al. (8). As seen in Table I addition of dithionite to hemoglobin in this treated buffer had only a very small effect on $K^{L}_{4,2}$.

Addition of the sulfhydryl reducing agents dithiothreitol and β-mercaptoethanol was found to protect hemoglobin samples against the increased dissociation by dithionite. In addition the effect produced by dithionite could be completely reversed by subsequent addition of these agents to hemoglobin samples. No concentration dependence was observed in the action of these agents between 3 and 200 μM. Bovine serum albumin (BSA) at a 140-μM concentration prevented the increase in $K^{L}_{4,2}$ when added before dithionite. If dithionite was added first BSA did not reverse the effect on $K^{L}_{4,2}$. Ethylenediamine acid (EDTA) neither protected hemoglobin from the effect of dithionite nor did it reverse the increase in $K^{L}_{4,2}$.

Addition of 0.7 μM $CuSO_4$ to a hemoglobin sample in Chelex 100 treated 0.1 M phosphate buffer had no effect on $K^{L}_{4,2}$. However, subsequent addition of dithionite to this solution produced an increase in $K^{L}_{4,2}$ similar to that shown in the first row of Table I. Addition of more $CuSO_4$ produced larger values of $K^{L}_{4,2}$ when dithionite was added. BSA and the sulfhydryl reducing agents showed the same protective and reversing properties for hemoglobin and $CuSO_4$ as was seen for hemoglobin in the untreated buffer. Recent experiments by Gray (14) in the presence of dithionite have also shown an increase in tetramer-dimer dissociation when Cu(II) and Zn(II) ions were added to hemoblogin solutions.

The efficacy of Chelex 100 in eliminating the effect of dithionite and the ability of Cu^{++} to work in concert with dithionite to increase $K^{L}_{4,2}$ provide indirect evidence suggesting that the unknown impurities are metal ions. The protective and reversing effects of sulfhydryl reducing agents suggest that binding of impurities to the β-93 sulfhydryl groups of hemoglobin may be involved in the increases in $K^{L}_{4,2}$.

The suggestion that traces of metal ions may be involved in the dithionite effect is consistent with the analysis listed on the phosphate buffers. Although they are very pure in terms of percentage, the large concentrations customarily used in experiments with hemoglobin may carry micromolar amounts of metals into the working solutions. If the dithionite effect is due to a reaction involving a small concentration of metal ions, the observation that $K^{L}_{4,2}$ tended to decrease somewhat when high concentrations of hemoglobin were used (though the measurement is more difficult) is not unexpected.

Finally, there is the question of whether the increase in the amount of rapidly recombining material after the addition of dithionite is really due to dimers. Our experiments alone cannot resolve this issue. Comparisons between determinations of $K^{L}_{4,2}$ using flash photolysis and ultracentrifugation with the same solutions (in the presence of dithionite) reported in reference 1 leave little doubt that dimers are involved in the effects reported here. Since the rapidly reacting material and the amount of dimers correlated well in these earlier experiments, it is reasonable to suppose that the increase in rapidly reacting material observed following addition of dithionite results from an increase in tetramer-dimer dissociation.

In view of the maximum sample-to-sample variation presented in Fig. 1 ($0.1\ \mu\text{M} \leq K^{L}_{4,2} \leq 0.3\ \mu\text{M}$) and the increases in dissociation produced by impurities our results may only impose an upper bound on the tetramer-dimer dissociation constant of carboxyhemoglobin in the absence of dithionite.

In summary, it appears that dithionite in concert with unknown impurities present in the buffer solutions produces a significant increase in the dissociation constant of carboxyhemoglobin. Either avoiding the use of dithionite or using one of the sulfhydryl reducing agents can result in an order of magnitude reduction in $K^{L}_{4,2}$. From the point of view of quantitative interpretation of experiments with carboxyhemoglobin, a factor of 10 decrease in K^{L2}_{4} can make it possible to neglect effects due to dimers. For example, for a 50-μM hemoglobin solution a $K^{L}_{4,2}$ of 2 μM corresponds to 18% of the heme being present in dimers whereas only 6% is present as dimers for $K^{L}_{4,2} = 0.2\ \mu\text{M}$.

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