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D. P. Flamig
University of Nebraska - Lincoln

L. J. Parkhurst
University of Nebraska at Lincoln

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Kinetics of the alkaline tetramer → dimer dissociation in liganded human hemoglobin: A laser light-scattering stopped-flow study*

(subunits/salt bridges)

D. P. FLAMIG AND L. J. PARKHURST

Department of Chemistry, University of Nebraska, Lincoln, Nebraska 68588

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ABSTRACT **The first-order dissociation of tetrameric HbCO to the dimer has been studied over the pH range 10.30–11.57 in a light-scattering stopped-flow apparatus using argon-ion laser excitation. The first-order dissociation rate constant varies from 0.25 sec^{-1} to 24.0 sec^{-1} over this pH interval. A semilogarithmic plot of k versus pH has a slope of 2.56 at pH 11.07, the midpoint. The pH dependence of the dissociation of the tetramer is consistent with progressive titration of α_1–α_2 and β_1–β_2 salt bridges. At pH 10.66, the dissociation rates of HbO_2, HbCO, methemoglobin, and HbCN vary less than 20% from their mean value. A study of the dissociation kinetics as a function of protein concentration allows one to obtain both association and dissociation rate constants, and hence equilibrium constants, for the tetramer ⇄ dimer reaction. In this manner, equilibrium constants were obtained on protein solutions with less than 15 sec of exposure to dissociating conditions.**

The tetramer–dimer equilibrium for human carbon monoxide-hemoglobin (HbCO) has been studied in the ultracentrifuge from pH 7.0 to 10.75 by Edelstein *et al.* (1) and Andersen *et al.* (2). Alkaline denaturation at high pH values during the long times required for the equilibrium studies (12 hr) did not allow the collection of sufficiently extensive data for fitting to proton equilibrium models because a plot of the equilibrium constant as a function of pH did not approach a plateau at high pH values. The rate of the spectroscopic change at 245 nm following a rapid pH jump in the stopped-flow apparatus was assigned to the tetramer → dimer process. These rate studies were at pH 10.6. A flow-flash study by Gibson and Antonini (3) at pH 7.0 and 0.25 μM initial concentration of tetramer gave a relaxation rate constant of 1.24 sec^{-1}. Observations of slowly varying spectroscopic changes at 429 nm of deoxygenated hemoglobin at pH 7.0 following rapid deoxygenation were interpreted by Kellett and Gutfreund (4) to give a dissociation constant for the tetramer of 1.35 sec^{-1}. In this paper we report the direct study of the dissociation process by laser light-scattering stopped-flow techniques following rapid changes in pH from 7.0 to values between 10.30 and 11.57.

Preparation

(*a*) Hemoglobin was prepared according to Geraci *et al.* (5) and was then equilibrated with 5 mM potassium phosphate buffer, pH 7.0, by passage over Sephadex G-25. The stock concentration was 2 mM in heme. The stock hemoglobin was centrifuged for 30 min (4°, 35,000 × *g*) before use to remove dust particles. Liganded forms were prepared in standard ways (6). (*b*) Dilute buffers (5 mM potassium phosphate) were used at pH 7.00 and concentrated buffers (0.1 M ϵ-aminocaproate–KOH) at pH 10.44–11.83. The buffers were centrifuged (4°, 35,000 × *g*, 30 min.) and then degassed in tonometers prior to kinetic runs. Kinetic runs were made by flowing the hemoglobin (20–180 μM in heme) in dilute buffer against an equal volume of various concentrated buffers. The mixed solutions after flow were collected and the pH was measured immediately.

Instrumentation and data analysis

The stopped-flow instrument has been described previously (7) and was modified for these studies by the replacement of the xenon arc light source, monochromator, and associated optics with an argon-ion laser (Lexel model 75, Palo Alto, CA), wavelength 488 nm. Data were collected as voltages by the on-line computer as described elsewhere (7), were reduced to the form of equation 3 of Goss *et al.* (7), and were then fitted by a single exponential decay model using the Fletcher–Powell (8) algorithm. For pH values below 10.8, equilibrium constants derived from our kinetic measurements as described below (see Fig. 1) were used to convert our relaxation constants to true dissociation rate constants.

Results and discussion

Over the pH interval 10.46–10.73 the dependence of the light-scattering relaxation constant on total hemoglobin concentration was studied. At each pH the relaxation followed first-order kinetics within experimental error; hence, one can write: $b^2 = k^2 + 4Hkk'$, in which b is the relaxation rate constant, k is the dissociation constant, k' is the association constant, and H is the total heme concentration. Fig. 1 shows a fit of the data to the equation, permitting a determination of $K_{4,2} = k/k'$ for 3 pH values. The function of Andersen *et al.* (equation 6 of ref. 2) was used for fitting these data and for obtaining $K_{4,2}$ values between pH 10.3 and 10.8 so as to convert relaxation rate constants to true dissociation constants. Kinetic light-scattering relaxation measurements can thus afford a rapid means for obtaining equilibrium constants with a precision equal to that from ultracentrifugation, provided that the dissociation reaction occurs in a single step. At pH 10.66, 21.5°, and 30 μM heme after mixing, the forms HbO_2, HbCO, methemoglobin, and HbCN had relaxation rate constants of 0.50, 0.49, 0.52, and 0.37 sec^{-1}, respectively. A statistical analysis of the data suggests that the relaxation rate constant for HbCN is significantly lower than the constants for the other three forms. This relative ligand insensitivity is in marked contrast to the behavior of the hemoglobin from *Lumbricus terrestris* (7) recently studied in this laborabory.

Fig. 2 shows the pH dependence of the dissociation rate constant for tetrameric HbCO. This figure summarizes 19 separate pH experiments using nine different preparations of

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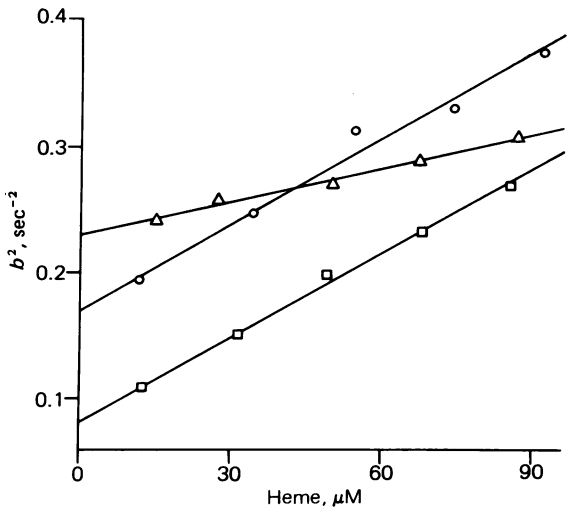

FIG. 1. A plot of b^2, the square of the relaxation rate constant, versus total heme concentration (after mixing) for the dissociation of human HbCO in a light-scattering stopped-flow apparatus. Buffers were 0.05 M ϵ-aminocaproate after mixing, temperature, 21.5°. □ = pH 10.46, ○ = pH 10.61, △ = pH 10.73. The figure sizes for each heme concentration correspond to 1 standard deviation in b^2 as obtained from a Fletcher–Powell fit of the average of from 15 to 20 reactions (200 points each) to a single exponential decay model with rate constant b.

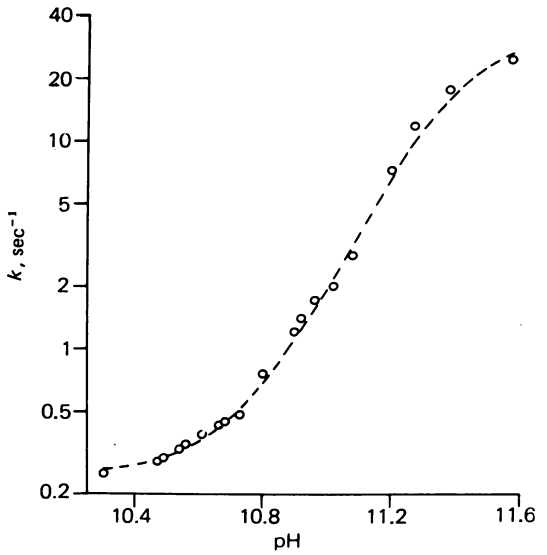

FIG. 2. A semilogarithmic plot of k versus pH for the alkaline tetramer → dimer dissociation of human HbCO at 21.5°. The broken line depicts the best fit to the data using a weighted least-squares procedure for the model

$$k = (k_0[H^+]^n + k_nK)/([H^+]^n + K)$$

in which k is the true dissociation rate constant, k_0 is the rate constant at low pH (*ca* pH 10.3), k_n is the rate constant for the dissociation of hemoglobin that has lost n more protons than has the species with rate constant k_0, and K is the equilibrium constant for the process

$$AH_n \rightleftarrows A^{n-} + nH^+.$$

hemoglobin, no one of which was older than 2 days at the time of the experiment. Each point represents the average of at least 5–10 kinetic runs, 200 data points per run. At pH 10.6 and 30 μM heme after mixing, our relaxation rate constant (0.45 sec^{-1}) agrees with that of Andersen *et al.* (2) obtained from tyrosine absorption changes. One can envisage two simple models for pH dependent dimer interactions. One model ("salt-bridge" model) assumes that titratable groups (most likely Lys-NH_3^+) on one dimer interact strongly with anionic groups on the other dimer; the other model assumes that the increasing negative charge on the tetramer, with increasing pH, leads to enhanced dissociation ("net-charge" model).

The broken line shows the best fit to the data (using a *weighted* least-squares Fletcher–Powell algorithm) for a simple salt-bridge model:

$$k = (k_0[H^+]^n + k_nK)/([H^+]^n + K).$$

Such a model implies the simultaneous dissociation of at least n acidic groups in the protein. Our best-fitting parameters are: n = 3.15, pK = 11.39, k_0 = 0.257 sec^{-1}, and k_n = 32.8 sec^{-1}. From symmetry, there must therefore be at least four titratable groups per tetramer involved in the dissociation reaction. Although to date the most extensive studies of salt-bridge interactions have been for human deoxyhemoglobin (9), it is of interest that Perutz *et al.* (10) reported the following salt bridges for α_1–α_2 and β_1–β_2 contacts in horse methemoglobin: Arg(141)CO_2^- ... $^+NH_3$Lys(127) and His(146)CO_2^- ... $^+NH_3$Val(1), respectively. These assignments are for horse methemoglobin and not for human HbCO at pH > 10. Goss and Parkhurst (11), in fact, report dissociation kinetics for horse HbO_2 that differ markedly from the results reported here. Garner *et al.* (12) have shown, however, that the pK of the NH_2-terminal NH_2 of Val(1) is 7.05 for human HbCO. Examination of a model of methemoglobin suggests the possibility of β_1His(146)CO_2^- ... $^+NH_3$Lys(132)β_2 interaction, possibly following a conformational change before pH 10.3. Perutz et al. (13) further proposed α_1–α_2 ionic interactions of Arg(141) and Lys(99)NH_3^+ groups with an internal anion in the central cavity.

One can also fit the dissociation kinetics over the ph range 10.3–11.0 quite well with a charged sphere model: $\ln(k/k_0) = -W^\dagger_{el} + W_{el} = RT\langle Z\rangle^2\ln(P)$, in which $\langle Z\rangle$ is the average net charge on the tetramer [the latter data are from the work of Orttung (14)], k_0 and P are adjustable parameters, † refers to a spherically symmetrical transition state with the same net charge but different radius from that of the initial state, and $W^\dagger_{el}$ and W_{el} are calculated from equation 26.30 of Tanford (15). Such a model, however, predicts a dissociation constant (k_0) of 0.02 sec^{-1} at pH 7. A dissociation constant from the work of Gibson and Antonini (3) of 0.85 sec^{-1} is obtained using their data and an equilibrium constant of 3.5 μM (dimer) for $K_{4,2}$ (6). Using our stopped-flow apparatus in the flow-flash mode (unequal syringes) and following the time course of the appearance of rapidly reacting hemoglobin generated by flash-photolysis following a 6-fold dilution at pH 7, we obtain a true dissociation rate constant of 1.0 sec^{-1} and $K_{4,2}$ = 3.4 μM (dimer), again assuming a one-step mechanism for dissociation. This discrepancy, however, could be attributable to a conformational change for liganded hemoglobin between pH 7 and 10. A recent spectroscopic study (16) might support such a change. On the other hand, a fit of the data to the model $\ln(k/k_1) = \Delta\langle Z\rangle^2\ln(P)$, in which k_1 is the observed dissociation rate constant at pH 10.3, results in net-charge values that are in marked disagreement with those calculated by Orttung (14). It is of interest to note that the dissociation rate constant at pH 7 is 4 times greater than that at pH 10.3, a result clearly in disagreement with the predictions of a net-charge model. A coulombic interaction such as α_1Tyr(42)O^- ... $^+NH_2$Arg(40)β_2 which is favored at high pH could account for such a change. We therefore conclude that the net-charge model alone is inadequate for treating the tetramer–dimer dissociation. We suggest that titration of α_1–α_2 and β_1–β_2 salt bridges in human HbCO plays a significant role in the tetramer-to-dimer dissociation process.

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