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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)

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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 α₁–α₂ and β₁–β₂ salt bridges. At pH 10.66, the dissociation rates of HbO₂, 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 Gutfeud (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 X 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 X 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 = k + 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 K₄₄ = 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 K₄₄ 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 HbO₂, 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 laboratory.

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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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 
P<sub>H</sub> dependent dimer interactions. One model ("salt-bridge" 
model) assumes that titratable groups (most likely Lys-NH<sub>3</sub>+) 
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 our protein. Our best-fitting parameters are: 
n = 5.15, pK = 11.39, k<sub>0</sub> = 0.257 sec⁻¹, and k<sub>n</sub> = 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 α1-α2 and β1-β2 contacts in horse methemoglobin: 
Arg(141)CO<sup>−</sup> ... +NH<sub>2</sub>Lys(127) and His(146)CO<sup>−</sup> 
+NH<sub>4</sub>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<sub>2</sub> that differ markedly from the results reported here.

Garner et al. (12) have shown, however, that the pK of the 
NH<sub>2</sub>-terminal NH<sub>2</sub> of Val(1) is 7.05 for human HbCO. 
Examination of a model of methemoglobin suggests the possibility 
of β1His(146)CO<sup>−</sup> ... +NH<sub>2</sub>Lys(132)β2 interaction, possibly 
following a conformational change before pH 10.3. Perutz et al. 
(13) further proposed α1-α2 ionic interactions of Arg(141) 
and Lys(99)NH<sub>3</sub>⁺ groups with an internal anion in the central 
cavity.

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**FIG. 1.** A plot of b², 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 (a) 1-aminocaproate after mixing, temperature, 21.5°C, 
O<sub>2</sub> = pH 10.46, O<sub>2</sub> = pH 10.61, Δ = pH 10.73. The figure sizes for each 
heme concentration correspond to 1 standard deviation in b² 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.

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**FIG. 2.** A semilogarithmic plot of k versus pH for the alkaline 
tetramer dimer dissociation of human HbCO at 21.5°C. The broken 
line lies closest 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<sub>0</sub> is the rate constant 
at low pH (ca pH 10.3), k<sub>n</sub> is the rate constant for the dissociation of 
hemoglobin that has lost n more protons than has the species with 
rate constant k<sub>n</sub> and K is the equilibrium constant for the process 

\[A_{Hn} = A^{-n} + nH^+\]
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