Biophysical Chemistry 3 (1975) 307–315

MECHANISM OF THE HEXAMER–DODECAMER REACTION OF LOBSTER HEMOCYANIN

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Received 29 April 1975

The overall forward and reverse rate constants for the hexamer–dodecamer reaction of lobster hemocyanin have been determined in 0.1 ionic strength glycine buffers at pH 9.6, at free calcium ion levels from 0.0031 to 0.0053 molar, at 25°C. Concentration-jump relaxation experiments in a stopped-flow apparatus were monitored by light scattered at 90°. The reaction is pseudobimolecular, and the overall forward rate constant bears virtually all of the calcium ion concentration-dependence, while the overall reverse rate constant is truly unimolecular. Four calcium ions appear to participate in the reaction between two hexameric molecules, and appear to become an integral part of the structure of the dodecameric molecule under these conditions.

1. Introduction

Evidence from ultracentrifugation [1] and kinetic experiments [2,3] have demonstrated that the interaction between two molecules of lobster hemocyanin, each containing six subunits, to form one molecule containing twelve subunits is both reversible and rapid in 0.1 ionic strength, pH 9.6 glycine buffers containing calcium ion. The ultracentrifuge studies [1] provided values for the apparent formation constant of the 12-subunit molecule (henceforth termed dodecamer) from two 6-subunit molecules (henceforth termed hexamer). Light-scattering temperature-jump measurements [2] first detected a step in a time range close to diffusion control. Stopped-flow kinetic studies [3] using the concentration-jump dilution relaxation technique [4] provided apparent forward and reverse rate constants for the overall bimolecular reaction, as well as derived apparent formation constants for dodecamer in good agreement with those from the ultracentrifuge. In the ultracentrifuge studies it was found that the formation of a molecule of dodecamer from two molecules of hexamer was accompanied by the net uptake of 4–6 calcium ions, when the free calcium concentration ranged from 0.0031 to 0.0053 moles/liter. In the stopped-flow studies [3], it was recognized that the derived rate constants were apparent values, characteristic in each case of the particular pH and calcium ion environment. However, the data obtained at pH 9.6 for two closely spaced calcium ion concentrations, 0.0031 and 0.0036 molar, indicated that most of the calcium ion concentration-dependence of the apparent equilibrium formation constant of dodecamer resided in the apparent overall bimolecular rate constant for reaction between hexamer molecules. This observation has an important bearing on possible elucidation of the reaction mechanism.

2. Theory

The first problem which occurs is that even in the case of a single reaction step between macromolecules, many liganded forms are possible for each macromolecular species. It is useful to state explicitly the implications of molecular weight and kinetic studies for the unraveling of multi-step reaction mechanisms involving the coupling of ligand binding with polymerization. We wish first to re-examine the meaning of the apparent equilibrium constant for formation of dodecamer from hexamer. Since the addition of a few calcium ions will have no experimentally discernible effect on the molecular weight of a single oligomeric form of protein as determined by ultracentrifugation, the equilibrium constant derived from ultracentrifugation may be written as the ratio of the sum of all

forms of dodecamer over the square of the sum of all forms of hexamer. Denoting by [A] the concentration of free hexamer, by $[A_2]$ the concentration of free dodecamer and by $[\bar{L}]$ the buffered free ligand concentration, we have the following relations:

$$A + L \rightleftharpoons AL, \quad K_1 = [AL]/[A][\bar{L}];$$
$$AL + L \rightleftharpoons AL_2, \quad K_2 = [AL_2]/[AL][\bar{L}];$$
$$\cdots\cdots\cdots\cdots\cdots\cdots\cdots\cdots\cdots\cdots$$
$$AL_{i-1} + L \rightleftharpoons AL_i, \quad K_i = [AL_i]/[AL_{i-1}][\bar{L}];$$

for hexamer, and

$$A_2 + L \rightleftharpoons A_2L, \quad K_1' = [A_2L]/[A_2][\bar{L}];$$
$$A_2L + L \rightleftharpoons A_2L_2, \quad K_2' = [A_2L_2]/[A_2L][\bar{L}];$$
$$\cdots\cdots\cdots\cdots\cdots\cdots\cdots\cdots\cdots\cdots$$
$$A_2L_{i-1} + L \rightleftharpoons A_2L_i, \quad K_i' = [A_2L_i]/[A_2L_{i-1}][\bar{L}];$$

for dodecameric species. The experimentally obtainable equilibrium constant from ultracentrifugation is termed the apparent formation constant of dodecamer from hexamer, given by

$$K_{app} = \frac{[A_2] + [A_2L] + [A_2L_2] + \ldots}{\{[A] + [AL] + [AL_2] + \ldots\}^2}$$

or, in terms of the previously defined ligand binding constants,

$$K_{app} = \frac{[A_2]\left(1 + \sum_{j=1}^{n}\prod_{i=1}^{j} K_i'[\bar{L}]^j\right)}{[A]^2\left(1 + \sum_{j=1}^{m}\prod_{i=1}^{j} K_i[\bar{L}]^j\right)^2}. \quad (1)$$

Here it has been assumed that there are n possible binding sites for ligand in a molecule of dodecamer, and m possible binding sites in a molecule of hexamer. It is noted that, although the intrinsic equilibrium constant $[A_2]/[A]^2$ is desired, it is not directly accessible from equilibrium molecular weights when polymerization is coupled with ligand binding. If one now differentiates the logarithm of K_{app} from eq. (1) with respect to the logarithm of the ligand concentration, $[\bar{L}]$, one obtains

$$\frac{\partial \ln K_{app}}{\partial \ln [\bar{L}]} = [\bar{L}]\frac{[A_2]\sum_{j=1}^{n}\prod_{i=1}^{j} jK_i'[\bar{L}]^{j-1}}{[A_2]\left(1 + \sum_{j=1}^{n}\prod_{i=1}^{j} K_i'[\bar{L}]^j\right)}$$
$$- 2[\bar{L}]\frac{[A]\sum_{j=1}^{m}\prod_{i=1}^{j} jK_i[\bar{L}]^{j-1}}{[A]\left(1 + \sum_{j=1}^{m}\prod_{i=1}^{j} K_i[\bar{L}]^j\right)}. \quad (2)$$

Inspection of these two fractions shows that the numerators represent the total number of moles of ligand bound to dodecameric and hexameric forms, respectively, while the denominators again represent total moles of protein present as dodecamer and hexamer, respectively. Hence the equation can be written in the form

$$\partial \ln K_{app}/\partial \ln [\bar{L}] = \bar{r}_{dodecamer} - 2\bar{r}_{hexamer} = \Delta\nu, \quad (3)$$

where $\bar{r}$ represents moles of ligand bound per mole of protein in the specified oligomeric form, and $\Delta\nu$ is the number of *excess* moles of ligand bound when two molecules of hexamer react completely to form one molecule of dodecamer. This is a perfectly general result completely independent of the microscopic mechanism of binding or polymerization. It assumes nothing at all about the relative strengths of binding or the possible addition or deletion of binding sites, nor can it provide any evidence at all to make a choice between such postulates. The result in eq. (3) is a straightforward special case of Wyman's theory of linked functions [5]. The functions being linked in the present example are the dimerization of a hexameric molecule and the binding of ligand by protein. It is to be noted here that if, as postulated in the previous ultracentrifuge study [1], a mechanism were shown by other means to hold, whereby two molecules of hexamer must first add a total of n additional molecules of ligand before being able to form a molecule of dodecamer, then it would also be true that $\Delta\nu = n$, since n simply means the number of excess ligand molecules bound during the completion of the reaction. On the other hand, equilibrium studies such as the determination of molecular weights with

the ultracentrifuge are in themselves wholly incapable of either verifying or disproving the validity of such an assumed obligatory mechanism of polymerization.

Consequently, resort is made to relaxation kinetic studies, which are intrinsically capable of making some distinctions between mechanisms. In the present case, two extreme mechanisms are proposed, aptly termed the ligand-mediated and ligand-facilitated mechanisms [6]. In the ligand-facilitated mechanism, it is assumed that two hexameric molecules can dimerize to a dodecamer, which is then stabilized by the binding of ligand. This is indicated as follows:

$$A + A \underset{k_d}{\overset{k_a}{\rightleftharpoons}} A_2 ; \qquad A_2 + nL \underset{k_{21}}{\overset{k_{12}}{\rightleftharpoons}} A_2L + (n-1)L$$

$$\underset{k_{32}}{\overset{k_{23}}{\rightleftharpoons}} A_2L_2 + (n-2)L \ldots \underset{k_{n+1,n}}{\overset{k_{n,n+1}}{\rightleftharpoons}} A_2L_n . \qquad (4)$$

The free ligand concentration is assumed to be buffered at $[\bar{L}]$ at all times. As a reasonable physical postulate, it is assumed that the rate constants for ligand binding (k_{12}, k_{21}, etc.) are all large compared to the rate constants for protein association and dissociation (k_a and k_d). This is equivalent to assuming that all ligand binding elementary steps equilibrate in times short compared to the overall reaction time for the association–dissociation process. The relaxation time for the latter process is then given by

$$\tau^{-1} = 4k_a[\bar{A}] + k_d/(1 + K_{12}[\bar{L}] + K_{12}K_{23}[\bar{L}]^2 + \ldots + K_{12}K_{23}\ldots K_{n,n+1}[\bar{L}]^n) . \qquad (5)$$

Here $K_{12} = k_{12}/k_{21}$, etc. The infinite time equilibrium concentration of hexamer, $[\bar{A}]$ and all relevant rate and equilibrium constants in eq. (5) are on the moles/liter concentration scale. For this mechanism, described by reaction scheme (4) and eq. (5), the relaxation time, τ, will become *longer* as the buffered free ligand concentration $[\bar{L}]$ is *increased*, because at a fixed total weight concentration of protein, $[\bar{A}]$ decreases with increasing $[\bar{L}]$, as does the second term in eq. (5). The apparent dissociation rate constant, equal to this entire second term, will therefore decrease with increasing ligand concentration, when this apparent rate constant is evaluated from relaxation kinetics experiments. The apparent bimolecular association rate constant, according to this mechanism, is k_a, which is independent of ligand concentration.

In the ligand-mediated mechanism, it is assumed that, in the present example, obligatory binding of two successive ligand molecules to each of two hexamer molecules must precede the formation of a molecule of dodecamer, as follows:

$$A + 2L \underset{k_{21}}{\overset{k_{12}}{\rightleftharpoons}} AL + L \underset{k_{32}}{\overset{k_{23}}{\rightleftharpoons}} AL_2 ; \qquad 2AL_2 \underset{k_{43}}{\overset{k_{34}}{\rightleftharpoons}} A_2L_4 . \qquad (6)$$

The general solution for the three relaxation time constants [7] for reaction scheme (6), without further assumptions, is to be obtained from the determinant

$$\begin{vmatrix} a_{11}-\tau^{-1} & a_{12} & a_{13} \\ a_{21} & a_{22}-\tau^{-1} & a_{23} \\ a_{31} & a_{32} & a_{33}-\tau^{-1} \end{vmatrix} = 0 . \qquad (7)$$

The coeffcients in the determinant are given by the following relations derived by linearization [7] of the classical rate equations:

$$a_{11} = k_{12}[\bar{L}] + k_{21} , \qquad (8)$$

$$a_{12} = -k_{21} , \qquad (9)$$

$$a_{21} = -k_{23}[\bar{L}] , \qquad (10)$$

$$a_{22} = k_{23}[\bar{L}] + k_{32} , \qquad (11)$$

$$a_{23} = -2k_{32} , \qquad (12)$$

$$a_{32} = -2k_{34}[\overline{AL_2}] , \qquad (13)$$

$$a_{33} = 4k_{34}[\overline{AL_2}] + k_{43} , \qquad (14)$$

and

$$a_{13} = a_{31} = 0 . \qquad (15)$$

The roots τ_1, τ_2 and τ_3 obey the following equations:

$$1/\tau_1 + 1/\tau_2 + 1/\tau_3 = a_{11} + a_{22} + a_{33} , \qquad (16)$$

$$1/\tau_1\tau_2 + 1/\tau_2\tau_3 + 1/\tau_1\tau_3 = a_{11}a_{22} + a_{22}a_{33} + a_{11}a_{33} - a_{23}a_{32} - a_{12}a_{21} , \qquad (17)$$

$$1/\tau_1\tau_2\tau_3 = a_{11}a_{22}a_{33} - (a_{11}a_{23}a_{32} + a_{12}a_{21}a_{33}) . \qquad (18)$$

The relationships in eqs. (16–18) can be readily verified by diagonalization, which is equivalent to expanding the polynomial $(1/\tau - 1/\tau_1)(1/\tau - 1/\tau_2)(1/\tau - 1/\tau_3)$

$= 0$, and comparing coefficients of like powers of $1/\tau$ with corresponding terms in eq. (7).

Two assumptions are now made to facilitate the extraction of the relaxation time constant for the protein interaction step. In the first assumption, the binding of the first ligand is taken to be appreciably faster than the binding of the second ligand ($a_{11}, a_{12} > a_{21}, a_{22}$). In the second assumption, which has both theoretical [8] and experimental justification, the terms in eq. (7) arising from the protein–protein interaction are taken to be quite small compared to those from ligand binding ($a_{11}, a_{12}, a_{21}, a_{22} \gg a_{32}, a_{33}$). With the aid of these assumptions, we obtain

$$\frac{1}{\tau_3} = 4k_{34}[\bar{A}] \frac{K_{12}^2 K_{23}^2 [\bar{L}]^4}{1 + K_{12}[\bar{L}] + K_{12}K_{23}[\bar{L}]^2} + k_{43} \quad (19)$$

Here again $K_{12} = k_{12}/k_{21}$ and $K_{23} = k_{23}/k_{32}$, and all concentrations and relevant rate and equilibrium constants are on the moles/liter scale. When $[\bar{A}]$ is expressed in mass/volume units, eq. (19) changes in form only in that the coefficient of the first term becomes 2 instead of 4 [3]. It is seen that for this mechanism, when $[\bar{L}]$ is small, the relaxation time for the slowest reaction step, τ_3, will *decrease* as the ligand concentration increases. Moreover, in any range of ligand concentration, the dissociation rate constant will be independent of the ligand concentration. These predictions are quite different from those derived from the ligand-facilitated mechanism, reaction scheme (4). A determination of relaxation times and rate constants over an appreciable range of ligand concentration should lead to a choice between possible mechanisms. Similar considerations in the magnesium-ion-coupled conformational changes of t-RNA in relaxation kinetics experiments have led to a choice between possible reaction mechanisms [9].

The second problem which occurs is that any bimolecular process which is found to have a rate constant many orders of magnitude slower than that predicted for a diffusion-controlled reaction cannot represent a single elementary step*. Any bimolecular reaction between identical spherical molecules should follow the Smoluchowski [10] and Debye [8] theories for diffusion-controlled reactions, with a rate constant independent of the molecular diameter. Deviations should be expected due to thermodynamic non-ideality [8], to steric requirements, and to reduction of such steric requirements by rotational diffusion of the reaction partners [11–13]. However, to within perhaps some two orders of magnitude, we should expect every elementary bimolecular reaction step to show a relaxation time characteristic of a diffusion-controlled reaction. In aqueous solutions at 25°C, this implies a maximum bimolecular rate constant of about $6.6(10)^9$ liters/mole/second. Under the conditions previously studied with temperature-jump light-scattering kinetics [2], this would predict a relaxation time of the order of 10 microseconds. Consequently, these extremely difficult observations in the time range 100–150 microseconds, with vanishingly small amplitudes [2], probably do represent the elementary bimolecular reaction step. The process being observed in the stopped-flow relaxation experiments, with relaxation times of the order of magnitude of a small multiple of ten seconds [3], is therefore a multi-step process, in terms of macromolecular interactions. Kirschner* has indicated that of the possible reaction mechanisms which can give rise to a slow pseudo-bimolecular process, one very plausible one is a diffusion-controlled elementary bimolecular reaction step to form an intermediate at a steady-state concentration, which then undergoes a slow conformational change to the final product. If the steady-state concentration is also very small, the observable amplitude of the elementary bimolecular step will also then become vanishingly small [14].

Omitting for the moment all ligand-binding steps, such a mechanism would be indicated by the scheme

$$A + A \underset{k_{21}}{\overset{k_{12}}{\rightleftharpoons}} A_2^* \underset{k_{32}}{\overset{k_{23}}{\rightleftharpoons}} A_2 \,. \quad (20)$$

The general procedure for evaluation of relaxation times for this mechanism indicates a fast step for which

$$1/\tau_1 = 4k_{12}[\bar{A}] + k_{21} \,, \quad (21)$$

and a slow step for which

$$1/\tau_2 = 4k_{23}[\bar{A}]/(K_{21} + 4[\bar{A}]) + k_{32} \,. \quad (22)$$

* Many of the thoughts in this section were developed directly by Prof. Kaspar Kirschner of the University of Basel in a seminar presented June 24, 1974 at the University of Connecticut. We are grateful to him for clarifying our thinking through that presentation, and for the stimulation which he provided.

Here $K_{21} = k_{21}/k_{12}$, the equilibrium constant for dissociation of the A_2^* intermediate. In the special case that the intermediate is at a steady state concentration, the slow relaxation time is obtained from the overall kinetic equation

$$d[A_2]/dt = k_f[A]^2 - k_r[A_2] , \tag{23}$$

where

$$k_f = k_{12}k_{23}/(k_{21}+k_{23}) , \tag{24}$$

and

$$k_r = k_{21}k_{32}/(k_{21}+k_{23}) . \tag{25}$$

This slow relaxation time is then given by

$$1/\tau = 4k_f[\bar{A}] + k_r . \tag{26}$$

Comparison of the general expression (22) with the more restricted relation (26) and the definitions of the overall rate constants k_f and k_r in (24) and (25) then shows that a steady-state concentration in A_2^* will be established, and an overall pseudo-bimolecular process will be found, provided that the following conditions are met: $K_{21} \gg 4[\bar{A}]$; $k_{23} \ll k_{12}$; $k_{23} \ll k_{21}$. The second condition is automatically fulfilled, since k_{12} must be the diffusion-controlled bimolecular rate constant. The first and third conditions are more specialized. The first will always be fulfilled at sufficiently low concentrations. The third condition will be fulfilled whenever the steady-state concentration of A_2^* is not large compared to $[\bar{A}]$ and $[\bar{A}_2]$, and whenever, in fact, the observed slow relaxation time is many orders of magnitude as long as expected for diffusion control, as in the present case. Another hypothetical mechanism, an obligatory slow conformational change of A to produce a reaction partner, can also slow the overall rate far beyond the expected diffusion-controlled rate. However, that mechanism does not lead to an overall pseudo-bimolecular slow relaxation time, as in eq. (26). Not only would the overall relaxation time *increase* with increasing total macromolecule concentration, but one of the two derived rate "constants" would vary with macromolecule concentration. Both of these predictions are contrary to experimental evidence in the present case [3]. It is noted that by eqs. (22) or (26), the overall forward rate constant is smaller than that for diffusion control by the factor k_{23}/k_{21}, which may be many orders of magnitude, while the overall reverse rate constant is the true dissociation rate constant of the final product, A_2.

2.1. *Final postulated reaction schemes*

The final postulated reaction schemes combine the ligand-binding postulates in either reaction schemes (4) or (6) with the macromolecular reaction scheme (20) leading to eqs. (22) and (26) for the slow relaxation time. In addition, one "compromise" ligand-binding mechanism is postulated, in which ligand can bind to macromolecule in several stages of the overall process. These reaction schemes, and their derived slow relaxation times are:

(a) *ligand-mediated*:

$$\begin{gathered} A + 2L \underset{k_{21}}{\overset{k_{12}}{\rightleftharpoons}} AL + L \underset{k_{32}}{\overset{k_{23}}{\rightleftharpoons}} AL_2 , \\ 2AL_2 \underset{k_{43}}{\overset{k_{34}}{\rightleftharpoons}} A_2L_4^* \underset{k_{54}}{\overset{k_{45}}{\rightleftharpoons}} A_2L_4 ; \end{gathered} \tag{27}$$

$$\frac{1}{\tau_{slow}} = 4\frac{k_{34}k_{45}}{k_{43}+k_{45}}[\bar{A}]\frac{K_{12}^2K_{23}^2[\bar{L}]^4}{1+K_{12}[\bar{L}]+K_{12}K_{23}[\bar{L}]^2} + \frac{k_{54}k_{43}}{k_{43}+k_{45}} ; \tag{28}$$

(b) *ligand-facilitated*:

$$\begin{gathered} 2A \underset{k_{21}}{\overset{k_{12}}{\rightleftharpoons}} A_2^* \underset{k_{32}}{\overset{k_{23}}{\rightleftharpoons}} A_2 , \qquad A_2 + 4L \underset{k_{43}}{\overset{k_{34}}{\rightleftharpoons}} A_2L + 3L \\ \underset{k_{54}}{\overset{k_{45}}{\rightleftharpoons}} A_2L_2 + 2L \underset{k_{65}}{\overset{k_{56}}{\rightleftharpoons}} A_2L_3 + L \underset{k_{76}}{\overset{k_{67}}{\rightleftharpoons}} A_2L_4 ; \end{gathered} \tag{29}$$

$$\frac{1}{\tau_{slow}} = 4\frac{k_{12}k_{23}}{k_{21}+k_{23}}[\bar{A}] + \left(\frac{k_{21}k_{32}}{k_{21}+k_{23}}\right) \times (1+K_{34}[\bar{L}]+K_{34}K_{45}[\bar{L}]^2+K_{34}K_{45}K_{56}[\bar{L}]^3 + K_{34}K_{45}K_{56}K_{67}[\bar{L}]^4)^{-1} ; \tag{30}$$

(c) *"compromise"*:

$$2A + 2L \underset{k_{21}}{\overset{k_{12}}{\rightleftharpoons}} 2AL \underset{k_{32}}{\overset{k_{23}}{\rightleftharpoons}} A_2L_2^* \underset{k_{43}}{\overset{k_{34}}{\rightleftharpoons}} A_2L_2 ,$$
$$A_2L_2 + 2L \underset{k_{54}}{\overset{k_{45}}{\rightleftharpoons}} A_2L_3 + L \underset{k_{65}}{\overset{k_{56}}{\rightleftharpoons}} A_2L_4 ; \qquad (31)$$

$$\frac{1}{\tau_{slow}} = 4\frac{k_{23}k_{34}}{k_{32}+k_{34}}[\bar{A}]\frac{K_{12}^2[\bar{L}]^2}{1+K_{12}[\bar{L}]}$$
$$+\left(\frac{k_{32}k_{43}}{k_{32}+k_{34}}\right)\frac{1}{1+K_{45}[\bar{L}]+K_{45}K_{56}[\bar{L}]^2} . \qquad (32)$$

All derivations of the expressions for the slow relaxation time have assumed ligand binding to be very fast, and have assumed a steady-state concentration of the (starred) intermediate. The differences between the predictions of eqs. (28), (30) and (32) are very real, both for the ligand concentration dependence of the forward and reverse rate constants and for the dependence of the observed reciprocal relaxation time on ligand concentration. The remainder of this paper concerns itself with the experimental test of these various proposed mechanisms by means of stopped-flow concentration-jump relaxation experiments at various controlled concentrations of free calcium ion.

3. Experimental

The concentration-jump experiments [3,4] were performed in a Durrum–Gibson stopped-flow apparatus [15] equipped with the commercially available fluorescence-scattering cell and photo-multiplier assembly for high response to scattering at 90°. All experiments were performed at 25°C by circulating water from a well-regulated water bath. A tungsten–iodine light source was used, with monochromator set at 436 nm, as this source provided best stability for slow recording. Solutions were prepared from freshly boiled distilled water to minimize air bubbles. The methods for obtaining and storing hemocyanin samples and the method for calculating the free calcium concentrations were the same as those described in the ultracentrifuge studies [1]. Protein solutions were measured at 5 to 6 different protein concentrations in pH 9.6, 0.1 ionic strength glycine buffer containing from 0.0031 to 0.0053 molar free calcium ion. Each solution was dialyzed against the corresponding buffer with which it was mixed in the stopped-flow experiment. Five volumes of protein were diluted with two volumes of buffer in each experiment. Additional details of the experimental procedure have been described earlier [3].

4. Results and data evaluation

It has been shown by numerous authors [16–19] that in the case of a single bimolecular reaction step involving equimolar concentrations of reaction partners, an unequivocal set of rate constants can be found without successive approximations and without resort to extraneous equilibrium data, by plotting the reciprocal of the square of the relaxation time versus the total concentration*. For the case of multiply coupled reactions, the situation will generally be too complicated to utilize this procedure. For the ligand-mediated mechanism, reaction scheme (27), we have deduced the following relationship:

$$\frac{1}{\tau_{slow}^2} = 4k_{45}^2\frac{k_{34}^2}{(k_{43}+k_{45})^2}\frac{K_{12}^2K_{23}^2[\bar{L}]^4}{(1+K_{12}[\bar{L}]+K_{12}K_{23}[\bar{L}]^2)^2K_{34}}$$
$$\times\left(\frac{c}{1+K_{45}}+\frac{[\bar{A}_t]}{K_{45}(1+K_{45})}\right)+k_{54}^2\frac{k_{43}^2}{(k_{43}+k_{45})^2} . \qquad (33)$$

Here c, the total protein concentration, $[\bar{A}_t]$ and k_{34} are all on the mass/volume concentration scale, and

$$[\bar{A}_t] = [\bar{A}](1+K_{12}[\bar{L}]+K_{12}K_{23}[\bar{L}]^2) \qquad (34)$$

is the total of all forms of hexamer. When the intermediate $A_2L_4^*$ is present in very low concentration, as well as in a steady state ($K_{45} \gg 1$), eq. (33) simplifies to

$$1/\tau_{slow}^2 = 4k_fk_rc + k_r^2 , \qquad (35)$$

* This method was also developed and described by us in a recent publication [3], without our having been aware of its earlier description.

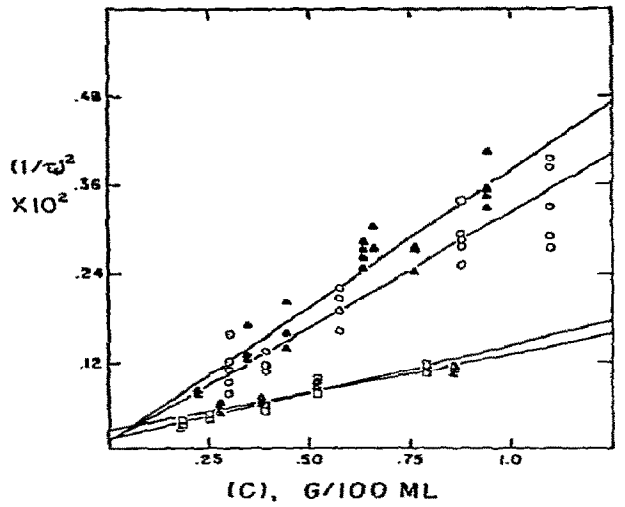

Fig. 1. Reciprocal of square of non-ideality-corrected relaxation time versus total protein concentration. Points are experimental and lines are least squares fit (see text). All data in 0.1 ionic strength glycine buffers at pH 9.6 and 25°C.
(▲): 0.0053 molar free calcium ion, 25 points;
(○): 0.0047 molar free calcium ion, 28 points;
(□): 0.0036 molar free calcium ion, 15 points;
(△): 0.0031 molar free calcium ion, 18 points.

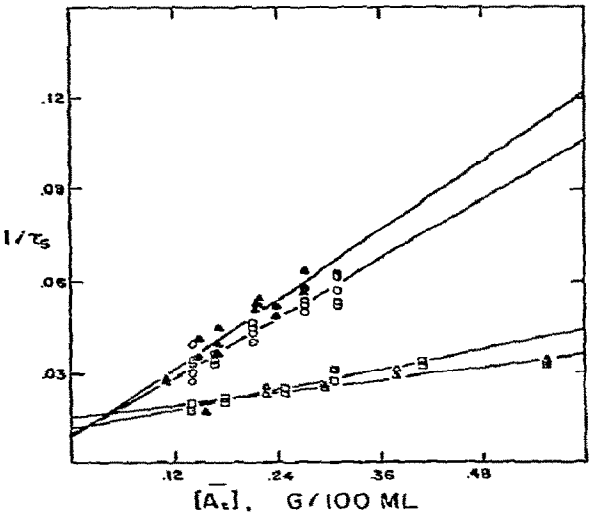

Fig. 2. Reciprocal of non-ideality-corrected relaxation time versus total hexamer concentration in all liganded forms (see text). All symbols and conditions as in fig. 1.

with

$$k_f = k_{45}K_{12}^2K_{23}^2K_{34}[\bar{L}]^4/(1+K_{12}[\bar{L}]+K_{12}K_{23}[\bar{L}]^2)^2 \quad (36)$$

and

$$k_r = k_{54}\,. \quad (37)$$

Hence a plot of $1/\tau_{slow}^2$ versus c gives an intercept whose square root is k_{54} and a slope/(intercept)$^{1/2}$ ratio which provides the same forward rate constant as eq. (28), subject to eq. (34) and conversion of concentration scales. This method of evaluation, used in our previous study [3] is therefore justified under this special set of conditions. The data to obtain relaxation times were obtained from least squares of the logarithm of the observed scattered light amplitude versus time after mixing [3]. Non-ideality coefficients obtained by Archibald ultracentrifuge experiments on non-interacting hemocyanin [1] were used to correct the relaxation times before plotting [3].

Plots of the reciprocal squares of non-ideality corrected relaxation times versus total macromolecule concentration are shown in fig. 1 for four different levels of free calcium ion. The intercepts provide values of the overall dissociation rate constants, which, when combined with the slopes, give values of the overall forward rate constants, according to eqs. (35)–(37). These overall rate constants were then used to calculate the equilibrium concentration of hexamer in all liganded forms, eq. (34). With the aid of these values of $[\bar{A}_t]$, a new set of least squares calculations for $1/\tau_{slow}$ versus $[\bar{A}_t]$ was made, according to eqs. (28) and (34), providing confirmatory values for the overall rate constants. The plots corresponding to these calculations are shown in fig. 2.

According to these observations, the primary data without any assumptions, the relaxation times, show a decrease with increasing concentration of free calcium ion. This is at once contrary to the predictions of the ligand-facilitated mechanism, reaction schemes (4) and (29), according to eqs. (5) and (30). Thus this mechanism is ruled out. The regular increase in the slopes in figs. 1 and 2, with increase in calcium ion concentration is in accord with the predictions of the ligand-mediated mechanism, as indicated in reaction schemes (6) and (27), and the independence of the in-

Table 1
0.1 ionic strength glycine, pH 9.6, 25°C

Free Ca^{2+} (M)	k_f (dl g^{-1} sec^{-1})	k_r (sec^{-1})	k_f/k_r (dl g^{-1})
0.0031 a)	0.0161	0.0164	0.98
0.0036 a)	0.0270	0.0120	2.25
0.0047	0.0799	0.0098	8.15
0.0053	0.0914	0.0101	9.05

a) Data taken from ref. [3].

tercepts on the calcium ion concentration is further support of this mechanism, as this is predicted in eqs. (19) and (28).

A summary of the overall forward and reverse rate constants for the hexamer–dodecamer reaction at pH 9.6 in 0.1 ionic strength glycine buffers at four different levels of free calcium ion is compiled in table 1. The results confirm that the overall rate constants for dissociation are relatively independent of free calcium ion concentration, while there is a strong dependence of the bimolecular association rate constants on calcium concentration over the same range. This effectively rules out not only the ligand-facilitated mechanism, but also the proposed "compromise" mechanism, which permits ligand binding to either hexamer or dodecamer, according to the predictions of eq. (32). According to the ligand-mediated mechanism, the slope of a plot of the overall pseudo-bimolecular rate constant should be given by differentiation of eq. (36):

$$\frac{\partial \ln k_f}{\partial \ln[\bar{L}]} = 4 - 2\,\frac{K_{12}[\bar{L}] + 2K_{12}K_{23}[\bar{L}]^2}{1 + K_{12}[\bar{L}] + K_{12}K_{23}[\bar{L}]^2} = \Delta\nu \,. \quad (38)$$

$\Delta\nu$ is simply the *excess* number of ligand ions bound per molecule of dodecamer over those bound per two molecules of hexamer, as may be verified by comparison with eqs. (2–3). Fig. 3 shows a plot of the data of table 1, according to eq. (38).

5. Discussion and summary

The slope $\Delta\nu$ of the plot in fig. 3 indicates that in the free calcium range 0.0031–0.0053 molar, an average of 3.5 calcium ions are added when two mole-

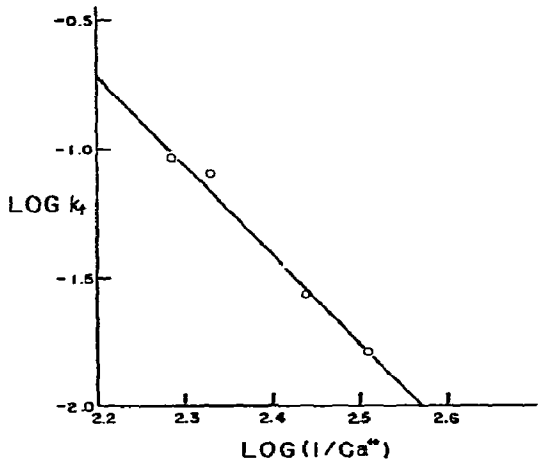

Fig. 3. Logarithm of overall forward rate constant versus logarithm of reciprocal of molarity of free calcium ion concentration. Points are experimental and line is least squares fit.

cules of hexameric hemocyanin unite to form one molecule of dodecameric hemocyanin, in close agreement with the equilibrium data obtained [1] from Archibald ultracentrifuge experiments. Moreover, essentially all of the calcium dependence previously observed in the equilibrium constant has now been found to reside in the pseudo-bimolecular rate constant, and essentially none in the unimolecular dissociation rate constant. This suggests that under the conditions of these experiments, there are four calcium ions which form an integral part of the structure of the dodecameric (whole) hemocyanin molecule — if any of these ions tries to leave the molecule, it can do so *only after* it has first dissociated in a unimolecular process into halves. The explicit number 4 written in eq. (38) arises entirely through the conscious mechanistic choice of two successive steps in which a total of two calcium ions is added to each hexamer molecule. Fig. 3 and its result $\Delta\nu = 3.5$ are derived directly from experimental data, however, and depend in no way on the choice of the number 4 in eq. (38). On the other hand, K_{12} and K_{23} are being interpreted as referring only to calcium ions binding to polymerization-mediating sites, in eq. (38). Since under the conditions of these experiments $\Delta\nu \approx 4$, *at least four specific* calcium-ion binding sites per whole hemocyanin molecule must be involved in a coupling with

polymerization. It is possible that more than four such sites are available, and that stable hemocyanin molecule structure can be attained even when they are not all filled. The fact that the dissociation rate constant is essentially independent of calcium ion concentration means that all such additional sites, if indeed they exist at all, are inaccessible to external calcium ions, once the dodecamer forms. On the other hand, at pH 8 and lower pH values, the ambient calcium ion level can be greatly decreased without loss of stability of the dodecameric form of the hemocyanin molecule, and ultracentrifuge experiments in this laboratory [20] suggested that $\Delta\nu$ may be as small as one calcium ion added per molecule of dodecamer formed at pH 8. We do not have any kinetic evidence to assess the mechanistic details of the situation under these conditions, but it is not possible to implicate four divalent metal ion bridges as being required for stability under all conditions, from our current results.

An additional set of kinetic experiments was performed in the present study, in analogous fashion to those just described, in which the ambient free calcium ion level was maintained 0.0036 molar, while the pH was varied from 9.4 to 9.7. Resulting data for overall forward and reverse rate constants indicated, however, that proton binding is neither as simple nor as specific as calcium ion binding. Both the overall forward rate constant (pseudo-bimolecular rate constant) and the overall reverse rate constant were found to be dependent on pH, as if some such compromise mechanism as that in reaction scheme (31) were operative. The data were not of sufficient quality to derive meaningful estimates of the numbers of protons involved: this requires a third derivative of observations.

Finally, it might be noted that for each of the reaction mechanisms considered, whether ligand-mediated, ligand-facilitated, or compromise, as in scheme (31), if one differentiates the logarithm of the apparent overall *equilibrium* constant (ratio of overall forward to reverse rate constants) with respect to the logarithm of the ligand concentration, one obtains precisely the same result, the right-hand side of eqs. (3) and (38). In other words, no matter which incorrect reaction mechanism is chosen, which is at variance with the results of kinetic experiments, the equilibrium behavior will explain the reaction mechanism equally satisfactorily (or equally meaningfully). The utilization of slow relaxation times, rather than equilibrium studies, to elucidate the mechanism of fast ligand binding steps has been reported by Kirschner et al. [21] in the case of glyceraldehyde-3-phosphate dehydrogenase.

The process is completely cooperative ($\Delta\nu = n$) if $K_{12} \to 0$ in eq. (38). The $k_{23} \gg k_{12}$ in reaction schemes (6) and (27), but this does not affect $1/\tau_{slow}$ if the ligand binding steps are relatively fast.

Acknowledgement

This study was made possible by National Science Foundation Grants GB29092 and GB 42899.

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