Proc. Natl. Acad. Sci. USA
Vol. 79, pp. 1825–1829, March 1982
Biochemistry

Conformational dynamics of a biologically active three-fragment complex of horse cytochrome *c*

(protein folding/concerted motion/interaction linkage/plurality of folding and unfolding pathways/modulation by temperature)

MARCEL JUILLERAT AND HIROSHI TANIUCHI

Laboratory of Chemical Biology, National Institute of Arthritis, Diabetes, and Digestive and Kidney Diseases, National Institutes of Health, Bethesda, Maryland 20205

Communicated by E. Margoliash, December 18, 1981

ABSTRACT **The conformational dynamics of a biologically active noncovalent complex containing three fragments, ferroheme fragment (1–25)H and apofragments (28–38) and [^{3}H](56–104) [or [^{3}H](39–104)], of horse cytochrome *c* has been studied with respect to kinetics and thermodynamics of dissociation. The rate of unfolding of the two-fragment complex ferro(1–25)H·(56–104) was also estimated. The results indicate that the ferrous three-fragment complex exhibits a higher frequency of dissociation–association with fragment (28–38) and a lower frequency of overall unfolding–folding at pH 7.0. In the presence of an excess of free (28–38) and below 30°C, unfolding of the ferrous three-fragment complex appears to occur by activation to the transitional state without a large change in conformation, followed by virtually simultaneous dissociation of all three of the fragments [without going through the complex (1–25)H·(56–104), which is a major intermediate for folding]. Above 30°C unfolding via the complex (1–25)H·(56–104) becomes detectable because the equilibrium between the two- and the three-fragment complex is highly temperature dependent. Thus, the relative probabilities of these two different ways of transition for unfolding are modulated by temperature. The observations suggest that the mode of activation of protein and hence the pathway for unfolding may vary depending on temperature. It is also suggested that the interatomic interactions binding the three fragments together in the ordered complex are linked to strengthen each other in the ground state.**

We have developed a biologically active three-fragment complex of horse cytochrome *c* as a model system in order to analyze the interatomic interactions maintaining the three-dimensional structure of proteins (1). The complex consists of heme fragment (1–25)H and two apofragments, (28–38) and (56–104); the ferric form of the complex exhibits an absorbance band characteristic of methionine ligation (1).

So far only two structural regions (residues 23–25 and 39–55) are known to allow cleavage without disrupting the characteristics of native cytochrome *c* (1, 2). As long as the discontinuity of the polypeptide chain is restricted to these two specific regions, the biologically active complexes can be formed from various combinations of two or even three fragments, including overlapping fragments (1, 2). These facts indicate that cleavage of the peptide bonds at these permissible sites does not disrupt the interatomic interactions critical for the native structure. Thus, the structure of the three-fragment complex, which has discontinuities of the polypeptide chain only at these two permissible regions, may also be maintained by interatomic interactions relevant for the native protein (1).

In the present study, we have investigated the kinetics and thermodynamics of dissociation of the fragments from the three-fragment complex in the ferrous state in order to assess the mode of activation of protein for unfolding on the basis of motion of the segments under physiological conditions.

MATERIALS AND METHODS

Heme fragment (1–25)H and apofragments (1–38), (28–38), (56–104), and (39–104) were prepared from horse heart cytochrome *c* (1, 2). Radioactive (56–104) and (39–104) were obtained by labeling both methionine residues 65 and 80 with [^{3}H]methyl iodide (Amersham) according to Jones *et al.* (3) after modification. [^{14}C]Phe-fragment (28–38) was synthesized by the solid-phase method (4) and purified by complex formation and gel filtration. The details of these methods will be described elsewhere. The labeled reconstituted complexes (1–25)H·[^{14}C]-(28–38)·(56–104) (970 dpm/nmol), (1–25)H·(28–38)·[^{3}H](56–104) (1150 dpm/nmol), and (1–25)H·(28–38)·[^{3}H](39–104) (23,800 dpm/nmol) were purified by ion-exchange chromatography on a column (0.7 × 30 cm) of SP-Sephadex C-25 (Pharmacia), using a linear gradient formed with 65 ml each of 0.03 M and 0.3 M ammonium acetate, pH 7.0 at 6°C (1).

Exchange of Fragment (28–38). Twenty microliters of 0.05 M potassium phosphate (pH 7.0) containing 23.8 nmol of labeled complex (1–25)H·[^{14}C](28–38)·(56–104) and 5 mg of sodium ascorbate was equilibrated at 3°C for 20 min and then mixed with 7.5 μl of 3.2 mM unlabeled (28–38). The fragment exchange mixture, consisting of 0.86 mM labeled ferrous complex and 0.87 mM free (28–38), was incubated for 10 min at 3°C. Then the complex and free (28–38) were separated by gel filtration on a column (0.5 × 18 cm) of Sephadex G-50 (superfine), using 0.05 M potassium phosphate (pH 7.0) at 3°C, to determine their radioactivities. The extent of fragment exchange was calculated as a function of time to obtain the rate constant of unfolding as described (5). Control experiments, run without addition of unlabeled (28–38), resulted in all radioactivity being associated with the complex.

Measurement of Unfolding of the Complex Ferro(1–25)H·(28–38)·(56–104). Measurement of exchange of fragment (56–104) (5) was hampered by its aggregation. This situation has led us to use of a trapping method. Because formation of the complex is much faster than dissociation (1, 5–7) and because ferrous two-fragment complexes unfold much more slowly (5) than does the ferrous three-fragment complex, our aim was to trap, in the presence of an excess of heme fragment (1–38)H and ascorbate, dissociated (56–104) as a stable ferro(1–38)H·(56–104).

In a typical run, to 31 nmol of labeled complex (1–25)H·(28–38)·[^{3}H](56–104), dissolved in 1 ml of 0.1 M potassium

The publication costs of this article were defrayed in part by page charge payment. This article must therefore be hereby marked "*advertisement*" in accordance with 18 U. S. C. §1734 solely to indicate this fact.

Abbreviations: Cytochrome *c* fragments are indicated by residue numbers in parentheses—e.g., (1–25). An H after the parentheses indicates heme.

phosphate (pH 7.0) and placed in a 15-ml conical tube, was added 250 μl of 1 M sodium ascorbate/0.1 M potassium phosphate, pH 7.0. Then the tube was flushed with N_2 and sealed with a rubber stopper. After equilibration for 15 min at given temperature, 600 μl of 1 mM (1–38)H, preequilibrated at the same temperature, was added and the tube was again flushed with N_2. This mixture of the labeled ferrous three-fragment complex and trapping heme fragment was incubated for a desired time at the same temperature. Then an aliquot (250 μl) was withdrawn and mixed with 45 μl of 5 mM apoprotein (1–104)/0.1 M ascorbate in order to bind the remaining (1–38)H (quenching of the trapping reaction). The mixture was then incubated at 37°C for 20 min. On the basis of control experiments, at this temperature the remaining complex ferro(1–25)H·(28–38)·[^{3}H](56–104) fully dissociates and (1–25)H thus released is trapped by (1–104) as ferro(1–25)H·(1–104). As a result, [^{3}H](56–104) of the remaining three-fragment complex is converted to the free state, which has been determined as follows.

Five microliters of 1% trypsin was added to the above treated mixture. After incubation for 20 min at 37°C the digestion was quenched with 10 μl of 2% soybean trypsin inhibitor. [Under these conditions, only free [^{3}H](56–104) is digested, while ferro(1–38)H·[^{3}H](56–104) is protected from digestion (2).] The digested material and the complex were separated by gel filtration on a column (0.5 × 50 cm) of Sephadex G-50 (superfine), using 0.03 M potassium phosphate (pH 7.0) at 6°C, to determine their radioactivities.

Fragment Exchange with the Complex Ferro(1–25)H·(28–38)·[^{3}H](39–104). In order to test the validity of the trapping method, a direct fragment exchange experiment was performed, using the complex ferro(1–25)H·(28–38)·[^{3}H](39–104), mainly because the complex containing either (56–104) or (39–104) is expected to show similar properties [the portion of residues 39–55 of ferro(1–25)H·(28–38)·(39–104) is presumably flexibly protruding (1)] and because of higher solubility of (39–104).

To 450 μl of a solution containing 15.2 nmol of labeled complex (1–25)H·(28–38)·[^{3}H](39–104) and 0–440 nmol of (28–38) that had been placed in a 15-ml conical tube, were added 15 μl of 1 M potassium phosphate (pH 7.0) and 155 μl of 0.1 M sodium dithionite (pH 7.0). This solution of the radioactive complex and another solution of 256 μM unlabeled (39–104) were separately incubated at 15°C for 15 min. Then a 200-μl aliquot of the latter solution was withdrawn and mixed with the former. The fragment exchange mixture, consisting of 18.5 μM radioactive ferrous three-fragment complex and 62.4 μM free (39–104), was incubated for the desired time. Then an aliquot (130 μl) was withdrawn, mixed with 50 μl of 0.02 M sodium dithionite (pH 7.0), and digested with trypsin (5 μl of 0.3% solution) for 15 min at 15°C. After addition of soybean trypsin inhibitor, the complex and digested fragments were separated to determine their radioactivities as described above. Control experiments were run without addition of the unlabeled fragment to correct for a partial digestion of the complex. On the basis of the extent of exchange thus determined as a function of time, the rate constants of unfolding were calculated (5).

Fragment Exchange with the Complex Ferro(1–25)H·[^{3}H](39–104). The procedure described above was modified for the two-fragment complex ferro(1–25)H·[^{3}H](39–104) in that the exchange reaction was quenched by addition of 13-fold molar excess of apofragment (1–38) to convert the above complex into the more stable ferro(1–25)H·(1–38)·[^{3}H](39–104).

Equilibrium Dialysis of the Complex Ferro(1–25)H·[^{14}C]·(28–38)·(56–104). A fully thermostatted apparatus consisting of five cells, each with two 570-μl cavities separated by a dialysis membrane (2000 M_r cutoff), was used for the experiments between 10°C and 35°C as described (8). All experiments were carried out with a buffer of 0.1 M potassium phosphate, pH 7.0/25 mM sodium dithionite containing 1 mM (56–104). Equilibrium with [^{14}C](28–38) across the membrane was attained within 5% in 30 hr at 5°C. One of the two cavities contained 300 μl of the above buffer and 1.2–10 μM ferro(1–25)H·[^{14}C](28–38)·(56–104) and the other 300 μl of the buffer alone. Under these conditions more than 98% of ferro(1–25)H·(56–104) is bound (7). The concentration of [^{14}C](28–38) in each cavity was determined by measuring radioactivity after dialysis for 40 hr.

The concentration of the three-fragment complex suitable for the experiment from 0 to 10°C was 0.27–0.40 μM. In this case dialyses were performed by using dialysis bags containing 15 ml of the sample solution immersed in 60 ml of the buffer in a thermostatted jacketed glass cylinder. After dialysis for 50 hr the radioactivities inside and outside the bag were determined.

RESULTS

Motility of Fragment [^{14}C](28–38) in the Complex Ferro(1–25)H·[^{14}C](28–38)·(56–104). The apparent dissociation constant K_d of fragment [^{14}C](28–38) was determined in the presence of sodium dithionite as a function of temperature by equilibrium dialysis (see *Materials and Methods*). The values for K_d and corresponding $\Delta G°$ are presented in Table 1. A van't Hoff plot was fitted to a straight line, resulting in the value for $\Delta H°$ equal to 34.2 kcal mol^{-1} (1 kcal = 4.18 kJ). The thermodynamic values thus obtained (Table 1) indicate a high degree of compensation of enthalpy and entropy for binding of fragment (28–38).

The dissociation–association reaction of fragment (28–38) is fast, because exchange of fragment [^{14}C](28–38) reached, in the presence of ascorbate, an equilibrium state within the time (19 min) limited by the method of measurement (see *Materials and Methods*) at 3°C (the dissociation rate constant is greater than 10^{-3} s^{-1}, the association rate constant is greater than 8×10^5 M^{-1} s^{-1}).

Unfolding of the Complex Ferro(1–25)H·(28–38)·[^{3}H](39–104). To know whether or not unfolding of the three-fragment complex occurs through the complex (1–25)H·[^{3}H](39–104), which is in equilibrium with the three-fragment complex (see above), exchange of [^{3}H](39–104) was measured at 15°C as a function of concentration of free fragment (28–38) present (see *Materials and Methods*). The rate constant of dissociation of [^{3}H](39–104) from the three-fragment complex in the ferrous form thus determined decreased with increase in the concentration of free (28–38) and reached a limiting value $1.7 \pm 1.0 \times 10^{-5}$ s^{-1} at

Table 1. Apparent dissociation constant (K_d) and the corresponding thermodynamic quantities for the dissociation of fragment [^{14}C](28–38) from the complex ferro(1–25)H·[^{14}C](28–38)·(56–104)

Temp., °C	K_d, nM	$\Delta G°$, kcal mol^{-1}	$\Delta S°$, cal mol^{-1} K^{-1}	$\Delta H°$, kcal mol^{-1}
0	0.8	11.4	83.4	34.2
5.4	1.85	11.1	82.9	34.2
10	11	10.3	84.4	34.2
9.9	30	9.6	86.9	34.2
15	30	9.8	84.7	34.2
20	140	9.2	85.3	34.2
25	130	9.1	84.2	34.2
35	1000	8.5	83.4	34.2

K_d was obtained by equilibrium dialysis; $\Delta G°$ was calculated on the basis of K_d; $\Delta H°$, from a van't Hoff plot; and $\Delta S°$, on the basis of $\Delta G°$ and $\Delta H°$.

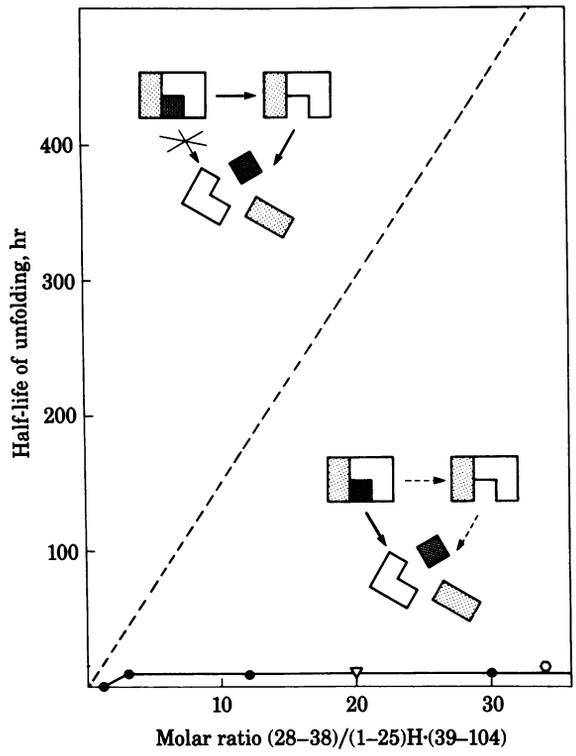

FIG. 1. Half-life for dissociation of [^{3}H](39–104) at 15°C as a function of the molar ratio, r, of fragment (28–38) added to ferro(1–25)H·(39–104). The dissociation rate constants, obtained from the fragment (39–104) exchange experiments, were $3.7 \pm 1.2 \times 10^{-4}$, $3.0 \pm 0.7 \times 10^{-5}$, $4.5 \pm 1.0 \times 10^{-5}$, and $1.7 \pm 1.2 \times 10^{-5}$ s^{-1} for r equal to 0.95, 3, 12, and 30, respectively (●). The open symbols represent the values obtained in the trapping experiments. The broken line represents the theoretical half-lives $\ln 2 \times [A_o/(k_{unf} \times K_d \times 3600)] \times r$, if ferro(1–25)H·(39–104) is an obligatory intermediate, assuming a dissociation constant, K_d, of 24 nM for fragment (28–38) (see Table 1), and a rate constant of unfolding, k_{unf}, of 1.0×10^{-2} s^{-1} for ferro(1–25)H·(39–104) at 15°C and that dissociation and association of fragment (28–38) are not rate limiting. A_o is the initial (total) concentration of complex (18.5 μM) (the equation is valid only when $r \gg 1$). The inserted schemes depict the dissociation reactions that would result in the corresponding half-lives.

15°C (Fig. 1). If the exchange of [^{3}H](39–104) took place only through the complex (1–25)H·[^{3}H](39–104), the rate constant of dissociation of [^{3}H](39–104) would have continuously decreased to a very low value with increase in the molar ratio of (28–38) added to the complex ferro(1–25)H·(39–104) under the conditions employed as shown in Fig. 1 (on the basis of half-life). Furthermore, the increase in the activation free energy for dissociation of [^{3}H](39–104) from ferro(1–25)H·(28–38)·[^{3}H](39–104) as compared with that from ferro(1–25)H·[^{3}H](39–104) is calculated to be 2.9 kcal mol^{-1} at 15°C, which is a fraction of the magnitude of free energy change (−9.8 kcal mol^{-1}) due to the binding with (28–38) and therefore less than that expected if [^{3}H](39–104) dissociates through ferro(1–25)H·[^{3}H](39–104). These results establish that fragment [^{3}H](39–104) dissociates from the ferrous three-fragment complex mostly without going through the two-fragment complex in the presence of excess of free (28–38) at 15°C.

Activation Parameters for Unfolding. The temperature dependence of the dissociation rate constant of fragment [^{3}H]-(56–104) from the complex ferro(1–25)H·(28–38)·[^{3}H](56–104) was determined in the presence of ascorbate by using a trapping method (see *Materials and Methods*) (Fig. 2). Control experiments by gel filtration have shown that heme fragment (1–38)H or (1–65)H or apoprotein (1–104) used as the trapping fragment

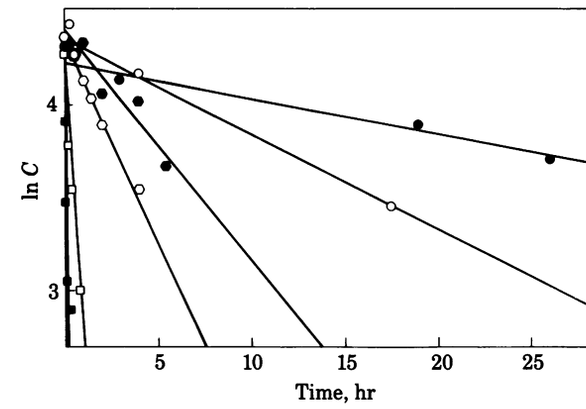

FIG. 2. First-order kinetics of unfolding of the complex ferro-(1–25)H·(28–38)·[^{3}H](56–104) as measured by trapping dissociated fragment [^{3}H](56–104). ●, 10°C; ○, 15°C; ●, 20°C; ○, 25°C; □, 30°C; ■, 37°C. C, percent of concentration.

binds with ferro(1–25)H·(56–104) to form a three-fragment complex [it is assumed that only the segment 28–38 of (1–38)H, (1–65)H, or (1–104) is incorporated into the ordered structure and the rest flexibly protrudes from the complex (cf. ref. 1)]. Thus, on the basis of the observations described above, only the direct dissociation reaction of [^{3}H](56–104) from the ferrous three-fragment complex would be measurable with the concentration of the trapping fragment employed (see Table 2). This assumption is supported by the observations that the dissociation rate constant ($1.7 \pm 1.0 \times 10^{-5}$ s^{-1}) at 15°C obtained by the fragment exchange method agrees within experimental error with that obtained by the trapping method (Table 2) and that addition of a 10-fold molar excess of free (28–38) over the three-fragment complex to the trapping system has resulted in no change (within experimental error) in the rate constant at 15°C and 20°C (Table 2). Neither substitution of (1–38)H by (1–65)H nor use of sodium dithionite in place of ascorbate significantly changed the rate constant. Furthermore, trapping of dissociated (1–25)H with apoprotein at 15°C resulted in a rate constant identical, within experimental error, to that determined by trapping of [^{3}H](56–104) with (1–38)H. Thus the dissociations of (1–25)H and [^{3}H](56–104) are concerted.

A modified Arrhenius plot of the data presented in Table 2 based on the absolute rate theory (9) is linear in the range from 5°C to 30°C (Fig. 3); an activation enthalpy of 34.6 ± 2.5 kcal mol^{-1} for dissociation of [^{3}H](56–104) can be calculated from these data. Above 30°C the plot seems to deviate from linearity.

Unfolding of the Complex Ferro(1–25)H·(39–104). Measurement of unfolding by exchanging fragment (39–104) in the complex ferro(1–25)H·[^{3}H](39–104) (see *Materials and Methods*) resulted in a rate constant of unfolding equal to $1.0 \pm 0.2 \times 10^{-2}$ s^{-1} at 15°C.

DISCUSSION

The present observations have revealed two distinct molecular motions for the ferrous three-fragment complex (1–25)H·(28–38)·(56–104): a high and a low frequency of association–dissociation reaction with fragments (28–38) and (56–104), respectively, at pH 7.0 below 30°C. Although the complex (1–25)H·(56–104) is an intermediate for formation of the three-fragment complex (1), the present results indicate that there is a mode of transition in which the ferrous three-fragment complex dissociates without going through the complex (1–25)H·(56–104) [e.g., in the presence of excess (28–38) and below 30°C]. Furthermore, in this mode of transition the dissociation

Table 2. Experimental conditions, rate constant, and activation free energy, enthalpy, and entropy for unfolding of the complex ferro(1–25)H·(28–38)·[^{3}H](56–104)

Temp., °C	Total conc. of three-fragment complex, μM	Conc. of trapping fragment, μM	α, %	k, $s^{-1} \times 10^6$	$\Delta H^\ddagger$, kcal mol^{-1}	$\Delta G^\ddagger$, kcal mol^{-1}	$\Delta S^\ddagger$, cal mol^{-1} K^{-1}
10.4	10	300[a]	0.004	6.8 ± 0.5	34.6	23.2	40.3
15	35	660[b]	0.005	16 ± 4	34.6	23.2	39.7
15	12	400[c]	0.007	12 ± 3			
15	16	300[d]	0.007	8.1 ± 5.0			
20	17	580[d]	0.013	50 ± 15			
20	53	600[b]	0.023	44 ± 2	34.6	22.9	39.2
25	60	660[b]	0.020	97 ± 2	34.6	22.4	40.2
30	48	660[b]	0.030	150 ± 100	34.6	23.0	39.6
30	48	660[e]	0.030	440 ± 40			
37	48	700[b]	0.143	6300 ± 700			

The rate constant of unfolding, k, was obtained by fitting the equation $A_{observed} = (A_o - B)e^{-kt} + B$ to the data by a nonlinear least squares routine (5). A_o is the initial concentration of ferro(1–25)H·(28–38)·[^{3}H](56–104), t is time, and B is the value at $t = \infty$. The percentage of complex ferro(1–25)H·(56–104) at time 0 in equilibrium with ferro(1–25)H·(28–38)·[^{3}H](56–104), α, was estimated by using the dissociation constants for fragment (28–38) (see Table 1) and taking into account that the region of residues 28–38 of the trapping fragments binds with the complex (1–25)H·(56–104) (see text). Activation parameters were determined, using the data obtained from 10°C to 30°C in the presence of ascorbate (see Fig. 3). In one experiment, dissociated heme fragment was trapped by apoprotein (1–104) and subjected to trypsin digestion at 15°C. The only labeled fragment digested, in this case, was dissociated [^{3}H](56–104).

[a] Trapping fragment, (1–38)H; reduction with ascorbate.
[b] Trapping fragment, (1–65)H; reduction with ascorbate.
[c] Trapping fragment, (1–104); reduction with ascorbate (see above).
[d] Trapping fragment, (1–38)H; reduction with ascorbate in presence of 10-fold molar excess of fragment (28–38).
[e] Trapping fragment, (1–65)H; reduction with 25 mM sodium dithionite.

rate constant with (1–25)H is the same within experimental error as that with (56–104), indicating that all three of the fragments dissociate (unfold) virtually simultaneously. With this dissociation reaction a high degree of compensation of activation enthalpy and activation entropy is associated (Table 2), a situation that is characteristic of unfolding of proteins (10, 11) and fragment complexes (5, 12).

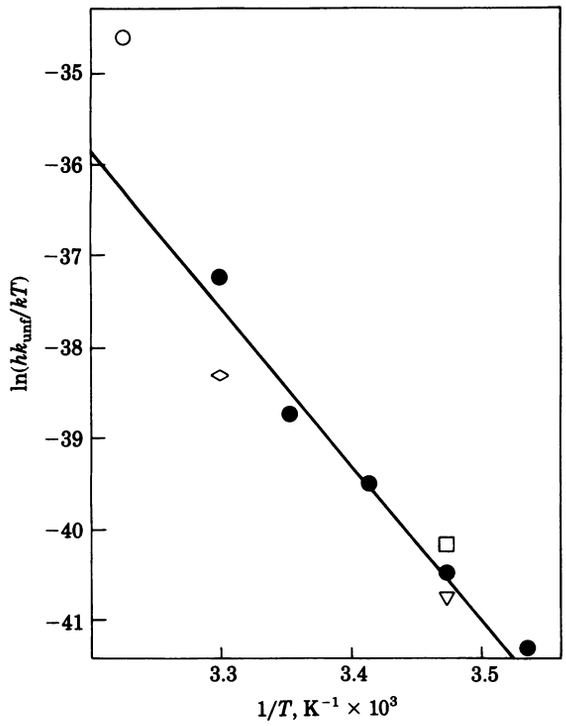

FIG. 3. Modified Arrhenius plot for unfolding of the complex ferro(1–25)H·(28–38)·(56–104). h, Planck's constant; k_{unf}, the rate constant of unfolding; k, the Boltzmann constant; T, absolute temperature. Symbols ○, □, ▽, and ◇ correspond to footnotes a and b, c, d, and e, respectively, in Table 2. The data from 10°C to 30°C (●) were fitted by a linear least squares procedure. Note that the point for 37°C (○) deviates from the line.

For this mode of unfolding the increase in the energy barrier (2.9 kcal mol^{-1} at 15°C) for the ferrous three-fragment complex as compared with ferro(1–25)H·(39–104) indicates that the interatomic interactions between ferro(1–25)H and (56–104) are strengthened upon binding with (28–38). Then, in turn, binding of (28–38) with ferro(1–25)H·(56–104) must also be strengthened by the interactions between (1–25)H and (56–104) (1).

The linearity of the Arrhenius plot for this mode of unfolding (below 30°C) (Fig. 3) indicates that change in heat capacity between the ground and the activated state is small ($\Delta C_p^\ddagger = \pm 130$ cal K^{-1} mol^{-1}) as estimated from the error in $\Delta H^\ddagger$ (±2.6 kcal mol^{-1}) (5, 10–12). Because exposure of nonpolar groups to water is expected to increase the heat capacity (13), the result may be interpreted as indicating that nonpolar groups buried within the structure are not largely exposed for the activated state (11). This is consistent with the hypothesis that the activation for unfolding would involve disruption of the specific cooperative interactions (coupling of interatomic interactions) operative throughout the three-dimensional structure without a large change in the fold (12, 14). The present observations that the activation is apparently followed by simultaneous disordering (dissociation) of all the segments further support this hypothesis.

This mode of unfolding is not reversal of folding through the two-fragment complex (1). However, such a folding pathway may not be the only one operative: simultaneous association of the three fragments could also occur with the rate constant greater than 5×10^{10} M^{-2} s^{-1} at 15°C (Fig. 4). One may also imagine that protein molecules could unfold through reversal of any of their folding pathways on the basis of experimental conditions. If these assumptions are correct, the present observations may be thought to reveal another pathway for folding, a pathway that is difficult to observe because of low probability (Fig. 4). Nuclease T stabilized after binding with ligands also

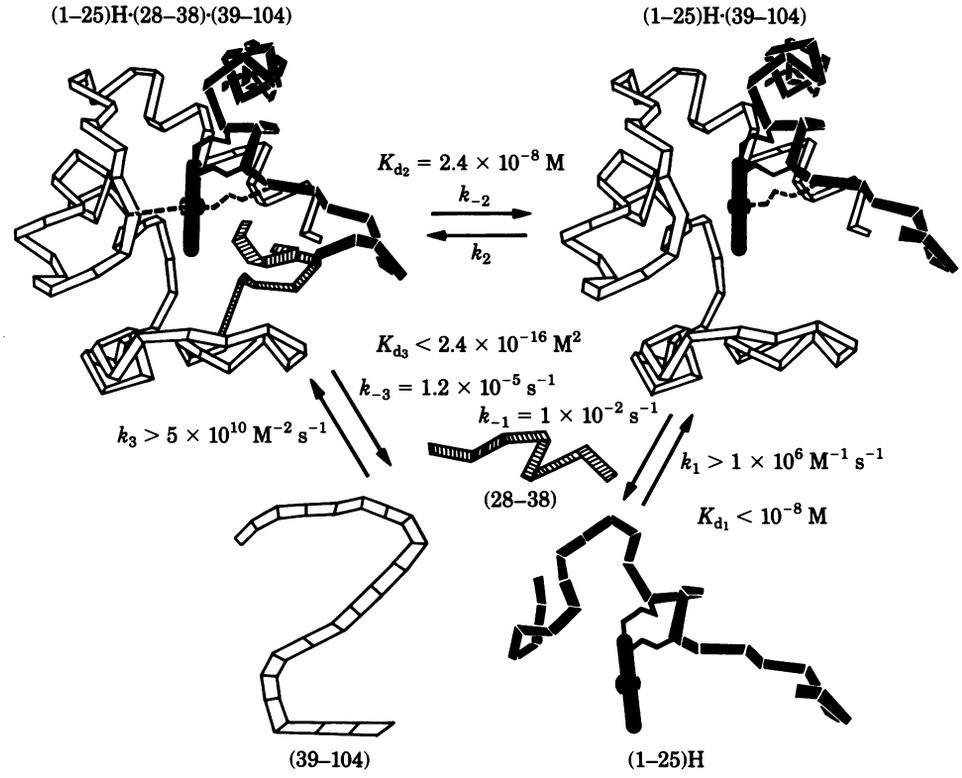

FIG. 4. Two pathways of folding and unfolding of the three-fragment complex with their respective rates and dissociation constants at 15°C (see text for the values for k_2 and k_{-2} at 3°C). The structural diagrams are after Almassy and Dickerson (15).

unfolds without going through dissociation of ligands when it is saturated with ligands (14).

The apparent deviation from linearity of the Arrhenius plot above 30°C (Fig. 3) may be explained by assuming that dissociation of [^{3}H](56–104) via the complex (1–25)H·(56–104) becomes significant above 30°C, because the complex (1–25)H·(56–104) in equilibrium with the ferrous three-fragment complex increases with increase in temperature (Tables 1 and 2). Such an equilibrium may be viewed as a transition between the two folded forms of different energy states. Thus, the relative probabilities of these two pathways of transition to the activated state, one with and the other without going through the two-fragment complex, are modulated by temperature. This suggests that the mode of transition for unfolding of protein may change as a function of temperature. In relation to these observations, it is interesting that the absorbance band at 695 nm of ferric cytochrome *c* decreases with increase in temperature before the heat-induced unfolding takes place (16), an observation that could be related to the increased mobility of a portion (e.g., residues 28–55) of the native protein.

1. Juillerat, M., Parr, G. R. & Taniuchi, H. (1980) *J. Biol. Chem.* **255**, 845–853.
2. Parr, G. R., Hantgan, R. R. & Taniuchi, H. (1978) *J. Biol. Chem.* **253**, 5381–5388.
3. Jones, W. C., Rothgeb, T. M. & Gurd, F. R. N. (1976) *J. Biol. Chem.* **251**, 7452–7460.
4. Merrifield, R. B. (1963) *J. Am. Chem. Soc.* **85**, 2149–2154.
5. Hantgan, R. R. & Taniuchi, H. (1978) *J. Biol. Chem.* **253**, 5373–5380.
6. Parr, G. R. & Taniuchi, H. (1979) *J. Biol. Chem.* **254**, 4836–4842.
7. Parr, G. R. & Taniuchi, H. (1981) *J. Biol. Chem.* **256**, 125–132.
8. Parker, D. S., Davis, A. & Taniuchi, H. (1981) *J. Biol. Chem.* **256**, 4557–4569.
9. Glasstone, S., Laidler, K. J. & Eyring, H. (1941) *The Theory of Rate Processes* (McGraw-Hill, New York).
10. Pohl, F. M. (1968) *Eur. J. Biochem.* **7**, 146–152.
11. Lumry, R. & Biltonen, R. (1969) in *Structure and Stability of Biological Macromolecules*, eds. Timasheff, S. N. & Fasman, G. D. (Dekker, New York), pp. 65–212.
12. Taniuchi, H. (1973) *J. Biol. Chem.* **248**, 5164–5174.
13. Cohn, E. J. & Edsall, J. T. (1943) *Proteins, Amino Acids and Peptides* (Reinhold, New York).
14. Taniuchi, H. & Bohnert, J. L. (1975) *J. Biol. Chem.* **250**, 2388–2394.
15. Almassy, R. J. & Dickerson, R. E. (1978) *Proc. Natl. Acad. Sci. USA* **75**, 2674–2678.
16. Schecjter, A. & George, P. (1964) *Biochemistry* **3**, 1045–1049.