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Properties of Modified Cytochromes

I. EQUILIBRIUM AND KINETICS OF THE PH-DEPENDENT TRANSITION IN CARBOXYMETHYLATED HORSE HEART CYTOCHROME *c*

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SUMMARY

Reduced (Fe^{+2}) carboxymethylated cytochrome *c*, Cm-cyt. *c*, undergoes a reversible pH-dependent transition with a pK of 7.16 at 20°. This pK is found to be nearly temperature-independent indicating that the over-all enthalpy for the transition is close to zero.

The kinetics of this transition have been investigated by the temperature jump technique. A single well resolved relaxation process (in the millisecond time range) is observed over the pH region of the static titrations. The amplitude of this relaxation at different wave lengths fits the statically derived difference spectrum between the alkaline and acid forms of the protein. Both the amplitude and the relaxation time (τ) are pH dependent; the over-all enthalpy of the process is estimated to be $\sim +1$ Cals per mole.

The observed behavior may be accounted for by a model in which a proton-linked conformational change in the protein is responsible for the spectral changes. It is suggested that the deprotonation of an ϵ-amino group of lysine (possibly that of lysine 79) is followed by the binding of this group to the ferrous iron to fill the vacant sixth coordination position. The observed spectral changes are attributed to the binding of a nitrogen atom to the iron.

The thermodynamic parameters governing the conformational part of the reaction are calculated on the basis of the above model and values of $\Delta H = -10$ Cals per mole and $\Delta S = -20$ e.u. are found. These values are discussed in the context of the binding of protein residue to the iron and the consequent changes in the crevice structure.

Under appropriate conditions (1-3) it is possible in horse heart cytochrome *c* to carboxymethylate the methionine residue at Position 80 which, in the native molecule, acts as the sixth coordination ligand of the iron (4). The product of the reaction has been shown to be capable of binding CO and O_2 at neutral pH (3), giving adducts which are spectrally similar to CO- or O_2-myoglobin and hemoglobin.

It has also been shown that the reduced form of the modified protein changes spin state going from high spin at acid pH to low spin at alkaline pH (3). Recent NMR measurements on the reduced material (5) indicate that at low pH the heme-iron is pentacoordinated and presumably out of the porphyrin plane similarly to what is known for deoxy myoglobin (6). On the other hand at neutral pH the abnormal temperature dependence of shifted resonances constitutes clear evidence that there is a mixture of two spin states under these conditions (5).

In view of the detailed knowledge on the structure of the system, it appeared worthwhile to undertake an investigation of the equilibrium and kinetics of ligand binding of the carboxymethylated protein. It soon became clear that the CO combination kinetics did not conform to a simple one step process and it was therefore necessary to study the system in greater depth. As a result, it has proved possible to explain quantitatively the ligand-binding process and the associated conformational changes taking advantage of the information now available on the three dimensional structure of native cytochrome *c* (7). The present communication deals with the kinetic and thermodynamic parameters governing the pH-dependent changes observable in the reduced protein.

EXPERIMENTAL PROCEDURE

Materials—Horse heart cytochrome *c* was purchased from Sigma Chemical Company (type III) and used without further purification. Di-methionyl-carboxymethyl cytochrome *c* was prepared essentially by the method reported by Schejter and George (1). A neutral solution 0.1 M in sodium cyanide, 0.2 M in bromoacetic acid, and 0.1 M in phosphate was prepared and cytochrome *c* was dissolved in this to give a final concentration of 10 mg per ml. The reaction was allowed to proceed for 24 hours at room temperature (25°) and then the reaction solution was exhaustively dialyzed against 0.02 M phosphate buffer for 2 days to remove the bromoacetate and bound cyanide. The modified cytochrome was stored at −20° until used. Details on the chemical characterization of this material will be reported in a following paper.

In brief it was found that the product of carboxymethylation was monomeric but consisted of a number (5 or 6) of electrophotometrically distinct species. Separation of these compo-

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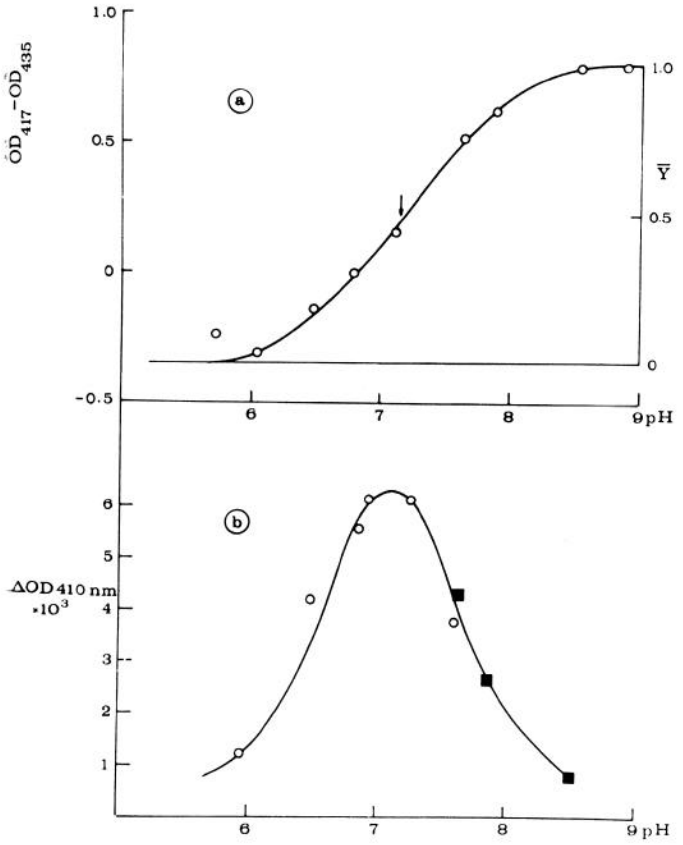

FIG. 1. *a*, spectrophotometric titration in the Soret region of 5×10^{-6} M reduced Cm-cyt. *c*. Buffers were 0.1 M phosphate below pH 8 and 2% borate above pH 8. Temperature 20°. *b*, changes in optical density at 412 nm observed in temperature jump experiments on reduced Cm-cyt. *c* plotted as a function of pH. Protein concentration = 2.5×10^{-6} M. ○, 0.1 M phosphate, ■, 2% borate.

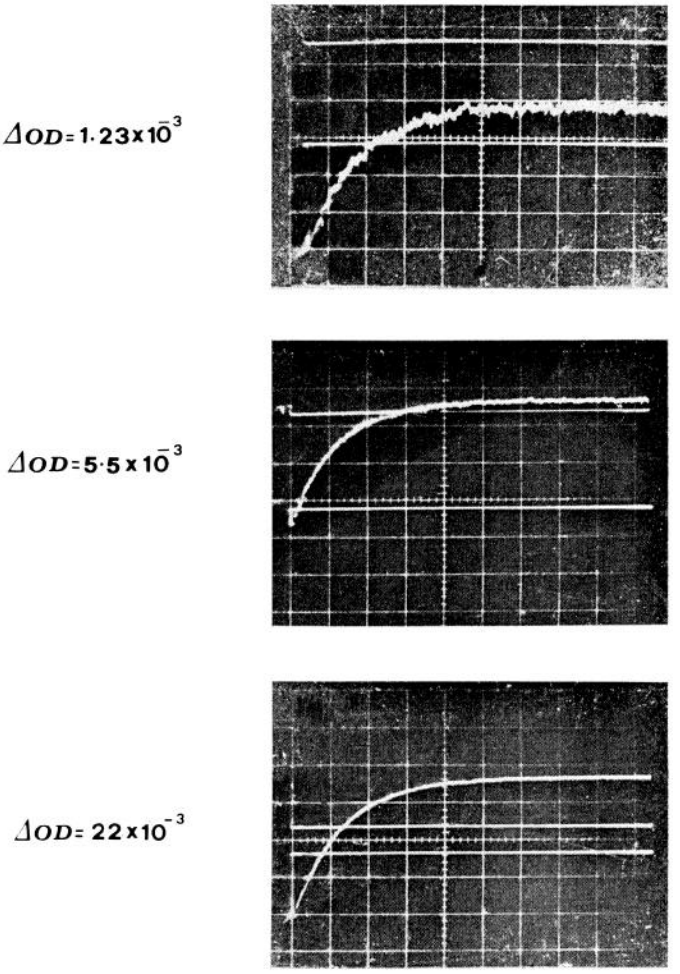

FIG. 2. Oscilloscope traces of temperature jump experiments on reduced Cm-cyt. *c* at pH 7, 0.1 M phosphate and 20°. Observation wave length = 412 nm. Sweep = 20 ms per grid division. Reduced Cm-cyt. *c* concentrations: from top to bottom *a*, 6.25×10^{-7} M; *b*, 2.5×10^{-6} M; *c*, 10^{-5} M.

nents by isoelectric focusing, however, allowed us to show that each of these species exhibited the same functional properties with respect to temperature jump experiments and ligand binding.

Dithionite was used throughout to maintain the protein in the reduced state (concentration ~0.5 mg per ml).

Methods—Spectrophotometry was carried out using a Cary-14. The pH values of all solutions were determined immediately after experimentation with a Radiometer model 4 pHmeter fitted with a type E5021a electrode capable of measuring the pH of very small volumes. Temperature jump experiments were carried out using an instrument built by Messanlagen Gesellschaft (Göttingen) through the courtesy of Prof. L. De Maeyer. Stopped flow measurements were carried out using a Durrum Instrument equipped with a 2 cm observation tube.

Modified cytochrome *c* concentrations were estimated spectrophotometrically using $\epsilon_M = 219{,}000$ at 414 nm for the CO derivative.

RESULTS

As reported previously (1, 3), the spectrum of reduced Cm-cyt. *c*[1] was found to be pH dependent. The transition, monitored in the Soret region, yielded a simple, completely reversible, titration curve over the pH range 6 to 10 with an n value of 1.0 and a pK of 7.16 at 20° (Fig. 1*a*). Investigations of the temperature dependence of this transition showed the over-all enthalpy of the process to be close to zero, values ranging from +1 to −1 Cals per mole being obtained. A second transition, with a pK ~ 5 was observed; however this was somewhat variable possibly because of side effects due to exposure of the protein to dithionite at low pH.

The kinetics of the transition were investigated extensively by the temperature jump technique. In phosphate buffer and at pH values close to the pK, a single well resolved relaxation effect was observed in the millisecond time range. The measured relaxation time (τ) was found to be independent of protein concentration over a range from 5×10^{-7} M to 10^{-5} M heme (see Fig. 2). The fact that upon increasing temperature the optical density at 412 nm increases (Fig. 1*b*) shows that the resolvable process reflects an increase in the alkaline form of the protein.

The amplitude of this relaxation was found to vary with pH and, as shown in Fig. 1*b*, a plot of the amplitude as a function of pH yields a symmetrical bell-shaped curve. Graphic integration of the area bounded by the data points in Fig. 1*b* in fact yields a smooth titration curve with $n = 1.0$ and pK = 7.12, which is almost the same as the value (7.16) obtained by static titration. Where the perturbation is small, this is expected if the kinetic and the static measurements reflect the same event.

As shown in Fig. 3, the wave length dependence of the ampli-

[1] The abbreviation used is: Cm-cyt. *c*, di-carboxymethylated cytochrome *c*.

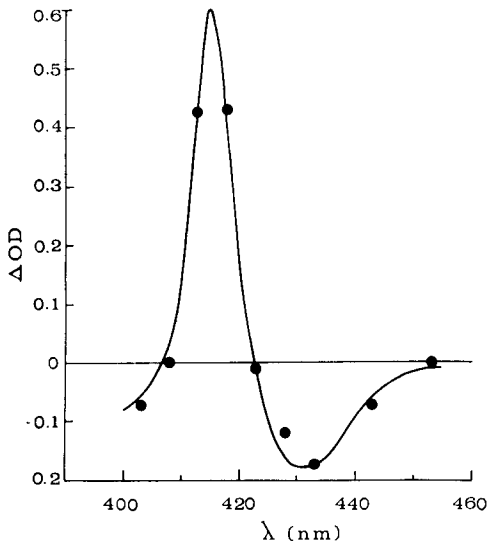

FIG. 3. Difference spectrum between the alkaline and the acid form of reduced Cm-cyt. *c*, obtained statically (——). Protein concentration = 4×10^{-6} M. ●, amplitude of the relaxation process as a function of wave length. The kinetic difference spectrum has been normalized to the static one at 433 nm. Temperature, 25°.

tude could be fitted after normalization at 433 nm, to the static difference spectrum between the alkaline and the acid forms of the protein.

The relaxation time was found to be pH dependent becoming shorter on increasing pH, as shown in Fig. 4*a* which gives $(1/\tau)$ *versus* pH. For a simple equilibrium between two forms of the protein, *e.g.*

$$\text{Acid form} \underset{k_b}{\overset{k_f}{\rightleftharpoons}} \text{Alkaline form} \qquad K = \frac{k_f}{k_b} = \frac{[\text{Alk}]}{[\text{Acid}]} \qquad (1)$$

the relaxation time is given by $\tau = (k_f + k_b)^{-1}$. It is therefore possible to derive, for any experimental condition, the interconversion rates, k_{forward} (k_f) and k_{back} (k_b), from a knowledge of the rate of approach to equilibrium and the equilibrium constant governing the distribution between the two forms. The former is obtained from temperature jump experiments and the latter from the titration curve measured statically. As shown in Fig. 4*b* log k_f depends linearly on pH over the range from 5.9 to 8.5, whereas log k_b is practically pH independent except below about 6, where the lower transition may come into play. It was not possible to explore the pH range above 9 as the signal becomes very small in this region (see Fig. 1*b*).

As is obvious from Fig. 4*a*, the relaxation time shows at all pH values a marked temperature dependence. It is interesting to note that at pH 7 a plot of log $(1/\tau)$ *versus* $1/T$ gives a straight line yielding an apparent activation energy of 19.3 Cals per mole (Fig. 5). One might expect such a straight line where the difference between activation energies associated with k_f and k_b is small.

Table I shows the relevant activation energies (ΔE_f and ΔE_b) calculated for three pH values on the basis of the data shown in Fig. 4*a*. The over-all enthalpy of the transition is positive throughout ($\Delta E_f - \Delta E_b = \Delta H = +1$–2 Cal per mole) and pH invariant within the experimental errors. This agrees with the direction of the displacement observed in temperature jump experiments, in which the alkaline form was always favored upon increase in temperature.

Yet another estimate of the apparent enthalpy for the transition was obtained using the value of K (Equation 1) derived from the static titration, in conjunction with the amplitude of the relaxation resulting from temperature jumps of different magnitudes. The value of ΔH obtained by this method is $\sim +1$ Cals per mole in good agreement with all other estimates. This, however, includes the heat of the phosphate buffer, itself very small (8). It is noteworthy that by changing the buffer system, *e.g.* by using Tris-HCl which has a large and positive enthalpy of ionization (8), the optical density change observed in a temperature jump experiment is reversed.

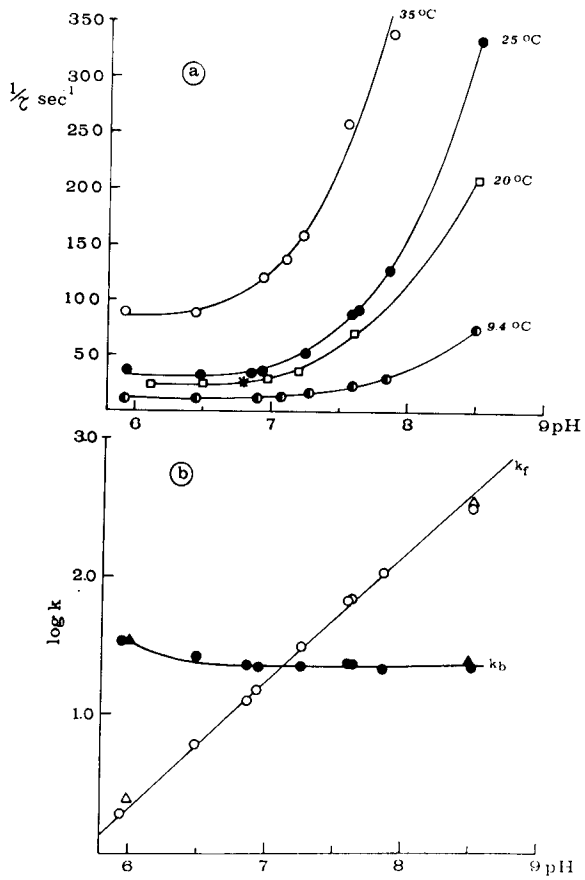

FIG. 4. *a*, Dependence of $1/\tau$ on pH at various temperatures. *, refers to the rate determined from pH jump experiments in the stopped flow. 0.1 M phosphate or 2% borate buffers. 2.5×10^{-6} M reduced Cm-cyt. *c*. *b*, Dependence of log k_b and log k_f on pH at 25°. The triangular and circular symbols refer to separate experiments.

The kinetics of the pH-dependent transition were also explored by pH jump experiments performed in a stopped flow apparatus. On mixing a lightly buffered solution of reduced Cm-cyt. *c* at pH 6 with a strong buffer at pH 9, no optical density change was observed between a few milliseconds and approximately 10 min. In agreement with expectations based on the temperature jump data (see Fig. 4*a*), this experiment implies that at pH 9 the transition was already completed within the dead time of the apparatus, *i.e.* about 4 ms.

The reverse experiment, of mixing the reduced protein at pH 9 with a strong buffer at pH 6, leads to an observable optical density change which corresponds to a first order process. As shown in Fig. 4*a* the apparent rate constant is in excellent agreement with the temperature jump results obtained under the same conditions.

In addition these experiments prove that no changes over and above those observed in the temperature jump experiments

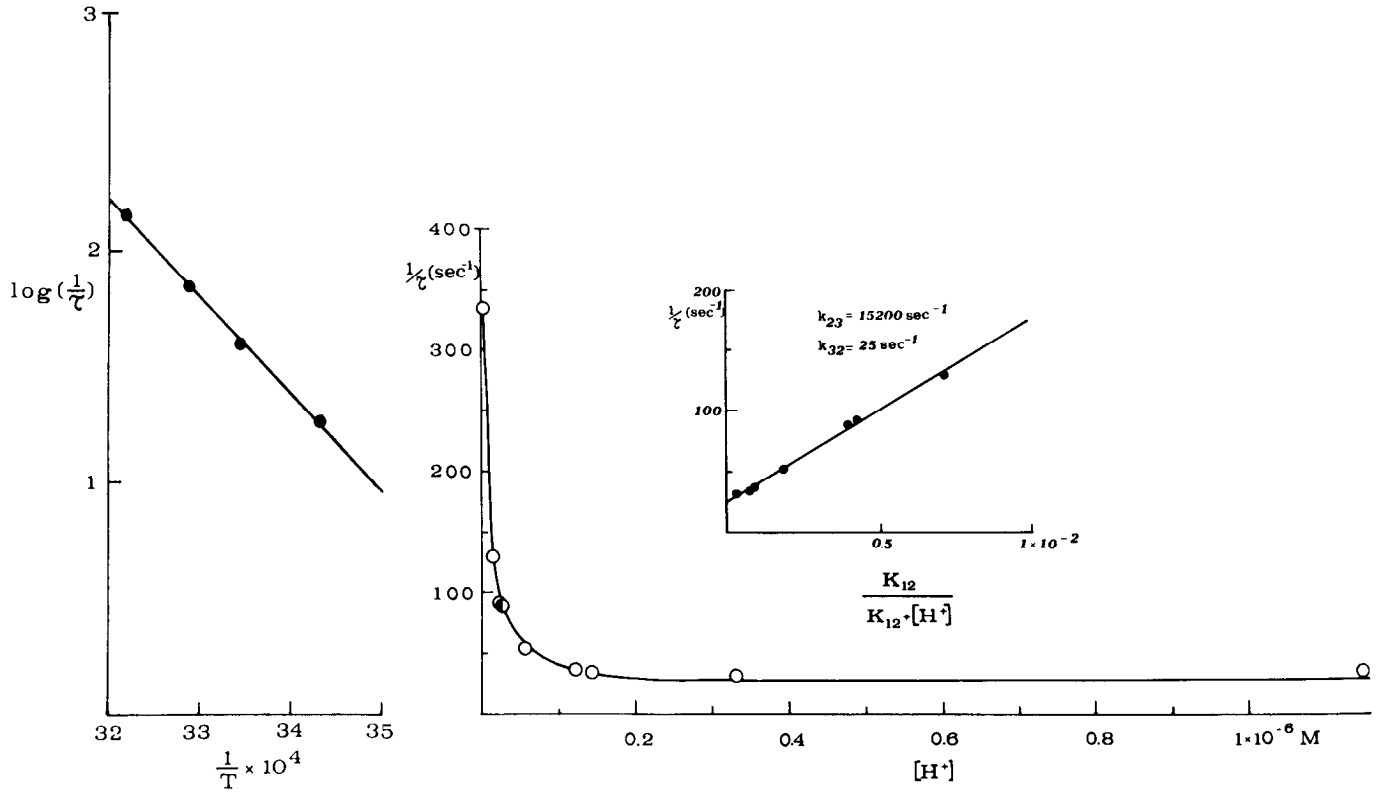

FIG. 5 (*left*). Dependence of $1/\tau$ on 1/T. 2.5×10^{-6} M Cm-cyt. *c*. pH 7 0.1 M phosphate.
FIG. 6 (*right*). Dependence of $1/\tau$ on [H] at 25°. 2.5×10^{-6} M Cm-cyt. *c*, 0.1 phosphate or 2% borate buffers. The insert shows $1/\tau$ plotted against $\{K_{12}/(K_{12} + [H])\}$ at 25° where K_{12} has been taken as 10^{-10} M.

TABLE I

Activation energies for the forward (k_f) and backward (k_b) rate constants in Equation 1

pH	ΔE_f	ΔE_b
	Cals/mole	*Cals/mole*
6	18.5	16
7	19	18
8	20.5	19

occur during the transition from the acid to the alkaline form of the protein.

DISCUSSION

The equilibrium data show that the reduced form of Cm-cyt. *c* undergoes a reversible pH-dependent spectral transition. In principle this transition may reflect either an ionization involving directly the chromophore, or a proton-linked structural change of the protein.

It seems clear from Figs. 1 and 3 that the kinetic events seen on perturbing reduced Cm-cyt. *c* pertain to the acid-alkali transition observed statically. The fact that only one relaxation effect is resolved may suggest that a single event, say a deprotonation, is responsible for the observed spectral change. There are, however, several arguments to suggest that this possibility is an oversimplification. A single step ionization process involving binding of a proton may be excluded on the basis of the relationship between $1/\tau$ and H^+ which, in the case of a single protonation, should be linear. The data in Fig. 6 clearly show this not to be so. On the other hand one step models involving binding of OH^- ions, such as: $PH_2O + OH^- \rightleftharpoons POH^- + H_2O$, where P is the protein, may be considered very unlikely for two reasons: (*a*) on spectral grounds, because OH^- and H_2O are extremely weak ligands for the ferrous iron and most probably are unable to produce the low spin character observed in reduced Cm-cyt. *c* at alkaline pH; (*b*) the rate constant for the dissociation of an OH^- for such reactions is typically 10^5–10^6 s^{-1} (9), whereas the comparable rate for the process involving reduced Cm-cyt. *c* (*i.e.* k_b) is only ~25 s^{-1}. In addition, the existence of a more complex situation is suggested by the fact that the apparent enthalpy associated with the transition ($\Delta H \simeq 1$ Cal per mole) is much too small for a group having a pK of approximately 7 (10).

One attractive alternative is to consider the existence of a proton-linked structural change of the protein which must be intramolecular, as the independence of $1/\tau$ on protein concentration rules out any proton-linked polymerization. A structural change has in fact been inferred to explain the pH-dependent change in the spin state of the heme in reduced Cm-cyt. *c* (3). The appearance at alkaline pH of a spectrum characteristic of a low spin heme complex reflects a change in the coordination state of the heme iron from penta to hexa coordinated *i.e.* the sixth position, which is free at low pH (like in myoglobin or hemoglobin), becomes filled at alkaline pH. As referred to in the introduction a similar conclusion has been drawn from recent NMR studies by Keller *et al.* (5). The most likely ligand to fill the vacant sixth coordination position of the iron is a nitrogen atom, possibly of an ε-amino group of lysine. Dickerson *et al.* (11) on the basis of x-ray diffraction studies, have in fact firmly suggested that lysine 79 is perfectly situated to coordinate with the iron in carboxymethylated cyt. *c*.[2]

[2] It may be of interest to quote Dickerson: "If this is done (Methionine (80) displaced as the 6th ligand) then lysine 79 can

Given the necessity of considering a proton-linked conformational change of the protein, the following general scheme may be written:

$$\begin{array}{ccc} & \mathrm{P} \rightleftharpoons \mathrm{P}^* & \\ \mathrm{H} + \downarrow\uparrow & & \downarrow\uparrow \quad + \mathrm{H} \\ & \mathrm{HP} \rightleftharpoons \mathrm{HP}^* & \end{array} \qquad (2)$$

where P and P* denote two conformational states of the protein, and H is the proton. Such a scheme seems unduly complex to account for the fact that only one relaxation time is observed on perturbing the system. This model may be simplified by considering one of the components to be present in negligibly small concentrations at equilibrium. An obvious choice, in the light of the above proposals that lysine 79 is involved as the sixth ligand, is to drop one or the other of the two protonated species, say HP*. The complete model reduces therefore to a simplified version:

$$\begin{aligned} \mathrm{HP} &\underset{k_{21}}{\overset{k_{12}}{\rightleftharpoons}} \mathrm{H} + \mathrm{P} \qquad K_{12} = \frac{k_{12}}{k_{21}} \\ \mathrm{P} &\underset{k_{32}}{\overset{k_{23}}{\rightleftharpoons}} \mathrm{P}^* \qquad K_{23} = \frac{k_{23}}{k_{32}} \end{aligned} \qquad (3)$$

where, according to the suggestion outlined above, HP is the pentacoordinated form with protonated lysine; P is a similar species with deprotonated lysine and P* is the hexacoordinated form in which the sixth coordination site is occupied by deprotonated lysine. Within this framework it seems reasonable to assign the spectral change to the transition from P to P*.

This model may be tested against the data. It may easily account for only one τ being observed, as the deprotonation process would be fast and presumably spectroscopically silent.

From the model in Equation 3 it may be shown that, where the proton is buffered and where deprotonation is fast compared with the conformational change, the slower relaxation time, which in our case is the only observed process, is given by:

$$(1/\tau) = k_{32} + \frac{k_{12}}{k_{12} + k_{21}[\mathrm{H}]} \qquad k_{23} = k_{32} + \frac{K_{12}}{K_{12} + [\mathrm{H}]} k_{23} \qquad (4)$$

where K_{12} is the intrinsic equilibrium constant for the deprotonation of HP. The general form of the dependence of $1/\tau$ on [H], predicted by Equation 4 is in fact observed, and is shown in Fig. 6.

While in principle Equation 4 may allow estimates of both k_{32} and k_{23} to be obtained, in practice only k_{32} can be determined unequivocally and any determination of k_{23} relies on assigning a value to K_{12}. On the basis of the above model, K_{12} may be given a value of 10^{-10} M, which is typical for the dissociation constant of ϵ-NH_3^+ in proteins (12). The insert to Fig. 6 shows that the data conform to Equation 4, using the above value for K_{12}.[3] The ratio of k_{23}:k_{32} (*i.e.* the equilibrium constant for the conformational change) can be determined from these data and is found to be $K_{23} = 610$. The conformational equilibrium, therefore, lies far to the right, the intermediate P being present in very small amounts.

be swung up until its amino nitrogen is exactly at the sixth ligand position of the iron. It is at least possible that the new ligand in the di-carboxy methylated protein is lysine 79."

[3] It may be noticed that the alternative two-steps model involving a proton binding process followed by a conformational change, *e.g.* P + H $\rightleftharpoons$ HP $\rightleftharpoons$ HP* is excluded on the basis of the relationship between $1/\tau$ and [H], which should be opposite to the one observed and reported in Fig. 6.

The apparent rate constants defined earlier (Equation 1) may be identified as follows:

$$k_b = k_{32} \quad \text{and} \quad k_f = \frac{K_{12}}{K_{12} + [\mathrm{H}]} k_{23}.$$

This latter relationship explains the linear dependence of log k_f on pH, shown in Fig. 3*b*, for where [H] is considerably higher than K_{12} (*i.e.* over most of the range explored taking $K_{12} = 10^{-10}$) than $\log k_f \cong \log K_{12} + \mathrm{pH} + \log k_{23}$.

The model in Equation 3 is, moreover, fully consistent with the equilibrium data. From this model it may be shown that the equation describing the titration curve has the form:

$$Y = \frac{K_{12}(K_{23} + 1)}{[\mathrm{H}] + K_{12}(K_{23} + 1)} = \frac{K'}{[\mathrm{H}] + K'} \qquad (5)$$

where Y is the fraction of protein in the alkaline conformation and $K' = K_{12}(K_{23} + 1)$. This equation predicts a simple titration curve, such as shown in Fig. 1, with an n value of unity. At the midpoint of the titration, where $Y = \frac{1}{2}$, $[\mathrm{H}] = K' = 6.9 \times 10^{-8}$ at 20° (*i.e.* pK′ = 7.16). Therefore, from the static titration, and taking $K_{12} = 10^{-10}$ M, K_{23} is found to be 690. Thus, once K_{12} has been chosen to correspond to the dissociation constant for lysine, a good agreement between the kinetic and equilibrium determinations of K_{23} is found.

Although possibly oversimplified, and probably not unique, the model in Equation 3 is seen to account quantitatively for the experimental data. Also, within its limitations, it allows one to calculate the thermodynamic parameters governing the conformational change P $\rightleftharpoons$ P*. Taking K_{12} as the dissociation constant for lysine, the enthalpy associated with the deprotonation may be assigned by reference to the literature (12) and this value, together with the over-all measured enthalpy of +1–2 Cal per mole, allows us to calculate all the remaining parameters. Table II lists the rate and equilibrium constants with their associated heats and entropies. The value of $\Delta H_{23} = -10$ Cals per mole is reasonable for an equilibrium which involves the formation of a bond, *i.e.* that between ϵ-amino group of lysine and the ferrous iron. Similarly the value of $\Delta S_{23} = -20$ e.u. might be expected for the coordination of the sixth ligand to the iron, as this would lead to a closing of the crevice structure and therefore to loss of flexibility in the molecule as a whole.

Evidence for slow ($k = 1.7$ s^{-1}) conformational changes in reduced native horse heart cytochrome *c* has recently been provided by Pecht and Faraggi (13). Unfortunately the relationship

TABLE II

Equilibrium and kinetic parameters for scheme of Equation 3 (at 25°)

Constant	Value	Enthalpy	Free energy	Entropy
		Cals/mole	Cals/mole	Cals/mole/°K
K_{12} (M)	10^{-10}[a]	+12[a]	+13.8[a]	−6[a]
K' (M)	6.9×10^{-8}	+1-2	9.9	−26
K_{23}	690[b]	−10	+3.9	−20
k_{23} (s^{-1}) (pH 7)	15,200	8.0*[c]	11.7*	−12.4*
k_{32} (s^{-1}) (pH 7)	25	18.0*	15.6*	+8.3*

[a] Values by reference to Tanford (12).

[b] K_{23} is here given the value determined from the equilibrium data and Equation 5.

[c] ΔH_{23}* was calculated from ΔH_{32}* and ΔH_{23}.

* Activation parameters.

between this conformational change and the somewhat faster process ($k = 25\ s^{-1}$) observed in reduced Cm-cyt. *c* is not at present clear.

It would be expected, on the basis of this model, that the functional properties (*e.g.* ligand binding) of Cm-cyt. *c* would depend on pH, reflecting the different structural properties of the different conformational states of the protein. Studies already completed on the kinetics of CO-binding to the reduced protein show this to be so, and will soon be reported.

REFERENCES

1. Schejter, A., and George, P. (1965) *Nature* **206,** 1150–1151
2. Stellwagen, E. (1968) *Biochemistry* **7,** 2496–2501
3. Schejter, A., and Aviram, I. (1970) *J. Biol. Chem.* **245,** 1552–1557
4. Dickerson, R. E., Kopka, M. L., Wenzial, J. E., Varnum, J. C., Eisenberg, O., and Margoliash, E. (1968) in *Structure and Function of Cytochromes* (Okunuki, K., Kamen, M. D., and Sekuzu, I., eds), published jointly by University of Tokyo Press and University Park Press
5. Keller, R. M., Aviram, I., Schejter, A., Wüthrich, K. (1972) *Fed. Eur. Biochem. Soc. Lett.* **20,** 90
6. Antonini, E., and Brunori, M. (1971) in *Hemoglobin and Myoglobin in Their Reactions with Ligands*, vol. 21, North-Holland Publishing Company, Amsterdam
7. Dickerson, R. E., Takano, T., Eisenberg, D., Kallai, O. B., Samson, L., Cooper, A., and Margoliash, E. (1971) *J. Biol. Chem.* **246,** 1511–1535
8. Bates, R. G. (1954) in *Electrometric pH Determinations*, J. Wiley and Sons, New York
9. Eigen, M., Kruse, W., Maass, G., and De Maeyer, L. (1964) *Progr. Reaction Kinet.* **2,** 287
10. Edsall, J. T., and Wyman, J. (1958) *Biophysical Chemistry*, Academic Press, New York
11. Dickerson, R. E., Takano, T., Kallai, O. B., and Samson, L. (1970) *Proc. Wenn. Green Symp.* , in press
12. Tanford, C. (1962) *Advan. Protein Chem.* **17,** 70
13. Pecht, I., and Faraggi, M. (1972) *Proc. Nat. Acad. Sci. U. S. A.* **69,** 902