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NMR study of the alkaline isomerization of ferricytochrome *c*

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The pH-induced isomerization of horse heart cytochrome *c* has been studied by ^{1}H NMR. We find that the transition occurring in D_2O with a pK_a measured as 9.5 ± 0.1 is from the native species to a mixture of two basic forms which have very similar NMR spectra. The heme methyl peaks of these two forms have been assigned by 2D exchange NMR. The forward rate constant (native to alkaline cytochrome *c*) has a value of 4.0 ± 0.6 s^{-1} at 27°C and is independent of pH; the reverse rate constant is pH-dependent. The activation parameters are $\Delta H^{\ddagger} = 12.8 \pm 0.8$ kcal·mol^1, $\Delta S^{\ddagger} = -12.9 \pm 2.0$ e.u. for the forward reaction and $\Delta H^{\ddagger} = 6.0 \pm 0.3$ kcal·mol^{-1}, $\Delta S^{\ddagger} = -35.1 \pm 1.3$ e.u. for the reverse reaction (pH* = 9.28). ΔH° and ΔS° for the isomerization are 6.7 ± 0.6 kcal·mol^{-1} and 21.9 ± 1.0 e.u., respectively.

Cytochrome *c*; Isomerization, alkaline; Kinetics; Activation parameters

1. INTRODUCTION

It has been known for many years that horse heart cytochrome *c* undergoes structural changes as a function of pH [1]. One of the best studied of these changes is the isomerization that occurs with an apparent pK_a of approx. 9.5 to give an alkaline form of cytochrome *c*. The structure of the alkaline form is still unclear, however, with a protein lysine [2], protein histidine [3] or hydroxide [4] proposed as an axial ligand replacing the methionine [5].

The kinetics of the conversion between the neutral species and the form above pH 9.5 have been the subject of study [6–8]. All of this work is complicated by the presence of additional forms of cytochrome *c*, which grow in as the pH is raised still further.

In view of the interest in the structure and motions of cytochrome *c* [9–11], especially in conjunction with the role of conformational change in electron transfer [12], we have reinvestigated the kinetics of formation of the alkaline species of cytochrome *c* using ^{1}H NMR spectroscopy. This work extends previous studies of the NMR spectrum of cytochrome *c* as a function of pH [13–17]. We report two new findings. First, the new species that appears with a pK_a of approx. 9.5 is not one but probably two species with very similar properties. Second, the forward reaction is independent of pH, but the reverse reaction is pH-dependent. Activation parameters for the forward and reverse rate constants have been measured.

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2. MATERIALS AND METHODS

Horse heart cytochrome *c* (type VI, Sigma) was dissolved in a $\mu = 0.1$ M phosphate buffer (99.96% D_2O). The pH of the solution was adjusted by titration with DCl or NaOD and monitored by an Orion Research 661 pH meter and an Ingold electrode which could fit in a 5 mm NMR sample tube. pH values were taken directly from the pH meter at room temperature without correction for the deuterium isotope effect or the temperature dependence of the pH of the buffer (which should be small for phosphate [18]) and are given herein as pH*.

^{1}H NMR measurements were performed on a Varian VXR-400. A 2D NMR pulse sequence [19] was used to visualize the exchange. In the 2D Fourier transformation, a sine bell weighting function was used for the f_1 and an exponential decay

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function for the f_2 dimension. Mixing times were selected using the method of Jeener and co-workers: $t_{mix} = [\ln((k + R)/R)]/k$ [19]. At 55°C, the exchange rate constant k is approx. 25 s^{-1} and R (T_1^{-1}) is approx. 10 s^{-1}; t_{mix} is therefore 0.05 s. In the kinetic studies, the peak of interest was inverted selectively by 180° and the magnetization was allowed to relax and exchange for a period $D2$ before the 90° observation pulse. Details of this technique may be found elsewhere [20]. It has been noted previously that the alkaline isomerization is not completely reversible [16]. However, we observed no changes in the kinetics of a given sample over the course of many days. It is expected that the species which cannot reform native protein are also not in exchange with native protein on the NMR time scale.

3. RESULTS AND DISCUSSION

Heme methyl groups in the basic form cytochrome *c* were assigned in a 2D NMR exchange experiment. Three 2D spectra were taken at room temperature with mixing times of 0.025, 0.05 and 0.12 s. At this temperature, the cross-peaks seen were due only to the nuclear Overhauser effect (not shown). When the temperature was raised to 55°C, both the exchange rate and T_1 increased, however. The exchange can be visualized from the cross-peaks between the heme methyl groups of the native species and those of the two basic forms (fig.1). No cross-peaks between the methyl groups of the two basic forms were observed even at 55°C, indicating that exchange between them is slower than exchange of each of them with the native species.

The 2D spectra allowed us to assign the 8-, 5- and 3-Me resonances of the heme ring (fig.2). The sequence of chemical shifts for the three methyl peaks changes from 8-Me > 3-Me > 5-Me for the native conformation [21] to 8-Me > 5-Me > 3-Me for both of the two basic conformations. This change implies that the electron density distribution in the heme plane is significantly different between the native and basic forms of ferricytochrome *c*. The ratio of the two basic forms B_1 and B_2 is a function of temperature. At 60°C, the methyl peaks of species B_1 are about 3-times as large as those of species B_2 (top trace in fig.2). However, when the temperature is lowered to 30°C the peaks due to species B_1 become smaller than those due to species B_2 (see the intensity change of the two 3-Me peaks, indicated as B3 in fig.2).

The rate constant of the reaction was measured at six different pH* values in the range 8.5–10 at 27°C. Fig.3 shows that the forward rate constant k_f is independent of pH with a value of 4.0 ± 0.6 s^{-1}. The reverse rate constant k_r increases from

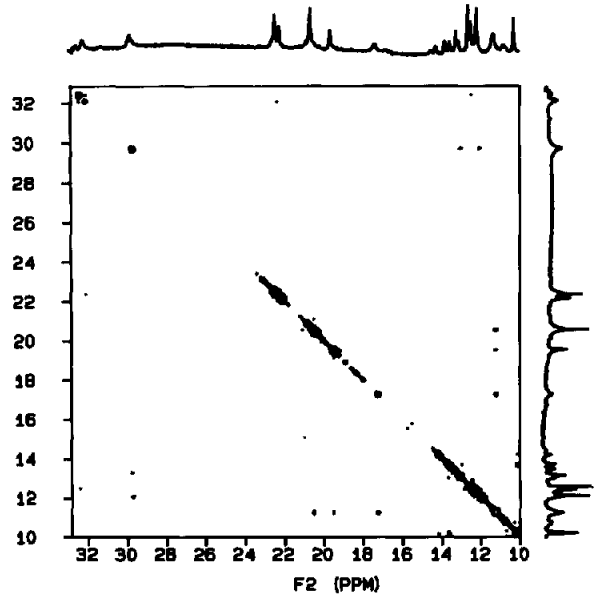

Fig.1. Downfield region of the 2D exchange spectrum of cytochrome *c* at 55°C and pH* 9.28 with mixing time 0.05 s.

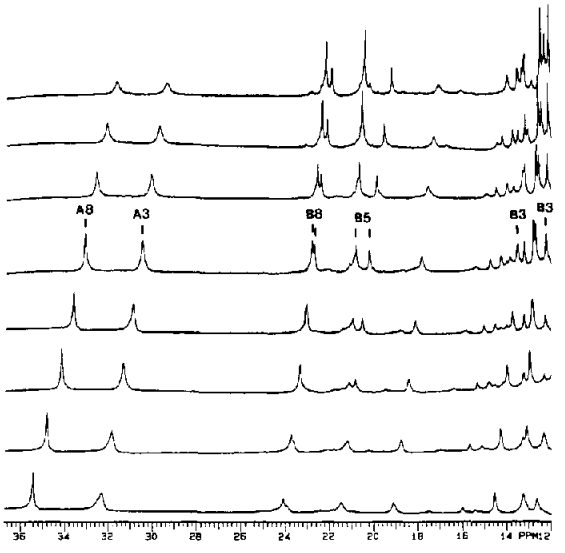

Fig.2. Stack plot of the downfield region of the cytochrome *c* spectrum as a function of temperature in 5°C intervals from 25°C (bottom) to 60°C (top) (pH* = 9.28 at room temperature). The labels indicate the assignments of the heme methyl resonances. A, native form; B, basic form.

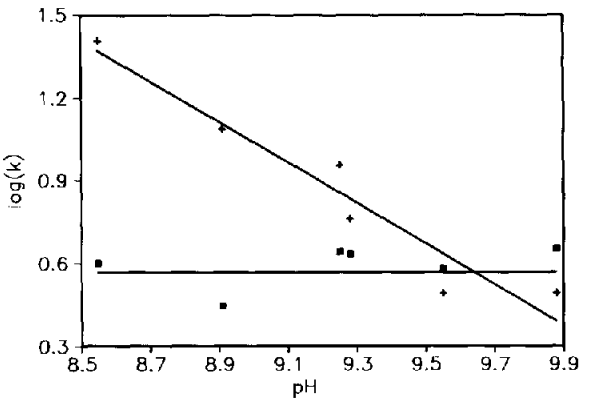

Fig.3. Forward and reverse rate constants as a function of pH*. (■) Forward reaction; (+) reverse reaction.

3 to 25 s^{-1} as the pH* decreases from 10 to 8.5. This observation suggests that the reverse rate involves protonation of a residue or axial ligand of the cytochrome ($k_r = k_r'[H^+]$). The pK_a value of cytochrome *c* in D_2O at 27°C was calculated as 9.5 ± 0.1 from the forward and reverse rate constants, k_f and k_r, using the expression: $pK_a = pH^* + \log(k_r/k_f)$; the same value was derived from the integrated areas of NMR peaks for the native and basic forms. This calculated pK_a value agrees with that measured by others [1–8,13–17,22].

To calculate activation parameters for this process, rate constants were measured at six different temperatures ranging from 27 to 55°C. Although

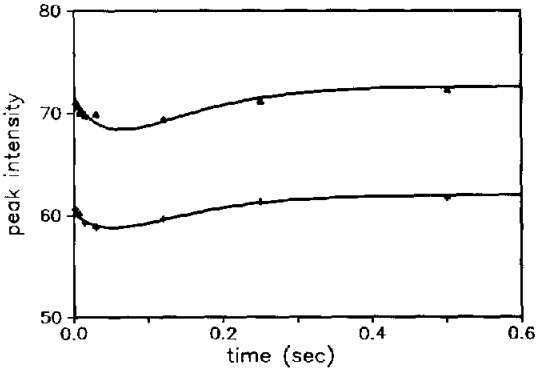

Fig.4. Intensity change of the 8-methyl peak in the two alkaline forms after the corresponding peak in the native form has been inverted by 180°. (+) Peak of species B_1 and (▲) peak of species B_2. The observation that the minimum intensity occurs at 0.03 s for both suggests that both exchange with the native form with the same rate constant (assuming similar t_1 values).

the native species is exchanging with two different forms which are not exchanging with each other, the kinetic behavior of the two basic forms is very similar (fig.4). Therefore, the kinetic analysis assumed a simple equilibrium A ⇌ B, where B is the sum of the two basic forms. The Eyring parameters of the forward reaction are $\Delta H^{\ddagger}$ = 12.8 ± 0.8 kcal·mol^{-1} and $\Delta S^{\ddagger}$ = −12.9 ± 2.0 e.u.; those of the reverse reaction are $\Delta H^{\ddagger}$ = 6.0 ± 0.3 kcal·mol^{-1} and $\Delta S^{\ddagger}$ = −35.1 ± 1.3 e.u. (fig.5, pH* = 9.28). The standard enthalpy and entropy change of this conformational change are ΔH° = 6.7 ± 0.6 kcal·mol^{-1} and ΔS° = 21.9 ± 1.0 e.u.

The rates observed here are faster than those measured in earlier optical studies. The most detailed study is that of Davis et al. [6] who observed an approach to equilibrium with a rate that increased with increasing pH and had a value of approx. 0.15 s^{-1} at pH 9.5. Their data was analyzed in terms of two species with a 695 nm band (presumably with the axial methionine ligated to the iron) and one without this band (presumably with the methionine not ligated to the iron); it appears that the optical and NMR studies do not visualize exactly the same species (or sums of species). Other related work involves measurement of the methionine off rate of cytochrome *c* via stopped-flow studies of the protein vs other ligands which replace the axial methionine [23–26]. In these experiments, the methionine off rate is 30–60 s^{-1}, faster than our observed rate of 4 s^{-1}. Again, the two types of studies may not visualize exactly the same forms of the protein. In addition,

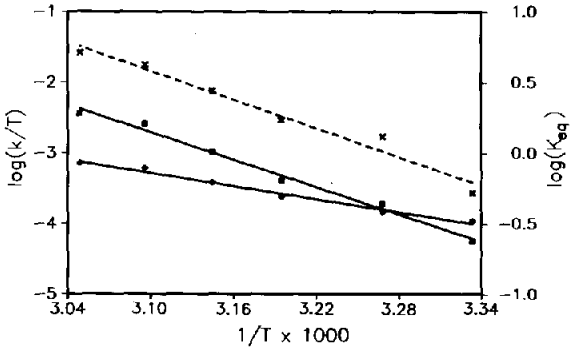

Fig.5. Eyring plot of forward and reverse rate constants over the temperature range 27–55°C at pH* = 9.28. (■) Forward reaction, (●) reverse reaction and (×) equilibrium constant.

the high ligand concentrations necessary for the stopped-flow studies may induce slight protein conformational changes, which could alter the methionine off rate.

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