Kinetics of electron transfer between cardiac cytochromes c_1 and c

(hinge protein/cytochrome c_1 -hinge protein-cytochrome c complex/ionic-strength-insensitive kinetic c_1 - c complex/stop flow at 10°C to -24°C/rapid-scanning spectrophotometry)

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Communicated by Martin D. Kamen, December 27, 1983

ABSTRACT Highly purified cytochrome c_1 , which consists of only one heme peptide and does not form a stable c_1 - c complex (c_1 -H- c complex), was used in studies of electron transfer between cytochromes c_1 and c . Results show that a stable and ionic-strength-sensitive c_1 - c complex (i.e., the c_1 -H- c complex) in the forms of the various oxidation states is not required, in contrast to the current belief of the participation of the complex in the electron transfer between cytochromes c_1 and c . A minimum mechanism for electron transfer between these two cytochromes is suggested in accord with the experimental results.

The molecular mechanism for electron transfer between cytochrome c_1 and cytochrome c remains to be clarified. The kinetics of this reaction were first studied (1) using purified preparations of c_1 (2) that contained two polypeptides, the cytochrome c_1 and the hinge protein. These could form a stable complex with cytochrome c (c_1 -H- c complex), sensitive only to ionic strength (3). The small hinge protein was required for the formation of this complex (4). Recently, electron transfer between cytochromes c_1 and c has been re-investigated by König *et al.* (5) using a c_1 preparation claimed to contain only one heme peptide (6). They suggested the stable c_1 -H- c complex as an intermediate in the electron-transfer mechanism (5).

We have studied the reaction of the electron transfer between cytochrome c and pure monomeric cytochrome c_1 containing only one polypeptide without the hinge protein (7, 8). Because of the very fast reaction between c_1 and c at room temperature, we have resorted to kinetic measurements at low temperatures where intermediate species might be seen. We have found that neither a simple mechanism of redistribution of charges between c_1 and c nor the explanation involving a stable complex (5) appears adequate.

MATERIALS AND METHODS

"One-band" monomeric cytochrome c_1 was prepared by an adaptation of the original method (4, 6, 9). The sample was highly purified without contamination by the hinge protein on the basis of various criteria (unpublished data). Cytochrome c was purchased from Sigma (type III) and used after further purification by Sephadex G-75 chromatography. Emasol 1130, similar to Tween 20, was a gift of Kao Soap (Tokyo). All other chemicals were obtained in the highest commercial grade available. Cytochromes were prepared in our laboratory in Albany, packed in dry ice, and shipped to Montpellier for low-temperature kinetic experiments. Cytochromes c_1^{2+} and c^{2+} were prepared by reduction with dithionite; cytochromes c_1^{3+} and c^{3+} were made by oxidation with solid potassium ferricyanide. Immediately before

use, reduced and oxidized cytochromes were prepared and passed through a Sephadex G-75 column. Experiments usually were carried out in 20 mM sodium cacodylate buffer (pH 7.4) at 2°C, where the pH profile of the rate constant for the oxidation of c_1^{2+} with c^{3+} is rather flat. For subzero temperature experiments, aqueous cacodylate buffer (pH 7.0) was diluted to 40% (vol/vol) ethylene glycol, the final concentration of the buffer being 20 mM. The protonic activities (p_{aH}) of this buffer were 7.4, 7.5, and 7.6 at 20, 0, and -20°C, respectively (cf. refs. 10 and 11).

Fast Kinetics and Rapid-Scanning Measurements. Stopped-flow and rapid-scan measurements were performed using a special stopped-flow mixing device (12), designed and built by INSERM (Montpellier), adapted to a Union Giken (model RA 415 and 401) fast-response spectrophotometer (13, 14). The temperature of syringes and mixing chamber of the stopped-flow were maintained at $\pm 0.1^\circ\text{C}$ with a circulating Haake bath model f3-Q. Rapid-scan absorption spectra were measured with a multichannel photodiode (maximum speed, $95 \times 10^3 \text{ nm sec}^{-1}$). The spectra were stored in a memory unit and then analyzed with a digital computer system. The kinetic responses were found to be first order, and the observed rate constants (k_{obs}) were determined from a nonlinear least-squares analysis using a computer fitting program. Single values of rate constants reported are the average of at least five experimental determinations. Kinetic constants have been estimated with an error not exceeding $\pm 20\%$.

Absorption spectra were obtained at 20°C or 2°C using either a Cary 219 or an Aminco DW-2 spectrophotometer equipped with a thermostatted cells holder (15).

RESULTS AND DISCUSSION

Reactions of c_1^{2+} with c^{3+} were measured during stopped-flow rapid mixing of the two cytochromes in 20 mM sodium cacodylate buffer (pH 7.4) at 2°C. A typical tracing of kinetics at 416 nm is shown in Fig. 1A. Fig. 1B depicts differential rapid-scan spectrophotometric measurements of the reaction at different times after mixing. The concentrations were 5.0 and 0.75 μM for c_1^{2+} and c^{3+} , respectively. The wavelength range used was from 352.5 to 447.5 nm. Optical difference spectra of the reaction mixture of $(c_1^{2+} + c^{3+}) - (c_1^{3+} + c^{2+})$ yielded an absorbance decrease at $416 \pm 1 \text{ nm}$. These observed spectral deviations are not those expected if the reaction proceeds simply as $c_1^{2+} + c^{3+} \rightleftharpoons c_1^{3+} + c^{2+}$.

A difference spectrum was experimentally obtained when comparing mixed equimolar concentrations of c_1^{2+} and c^{2+} with the same concentration of these two cytochromes in separate tandem cells (Fig. 2, spectrum A). Another difference spectrum was seen when both cytochromes in the oxidized form were mixed and compared with these two cyto-

FIG. 1. Electron-transfer reaction between c_1^{2+} and c^{3+} . (A) Kinetic tracing at absorption maximum (416 nm). (B) Rapid-scan spectrophotometric measurements of the reaction of ferrocytochrome c_1 with ferricytochrome c . The spectra are the computer-calculated difference spectra against reference to the final spectrum (96 msec). Scanning time was 2 msec for each spectrum. Numbers on the figure indicate the elapsed time after mixing, in msec; the concentrations of c_1^{2+} and c^{3+} are $5.0 \mu\text{M}$ and $0.75 \mu\text{M}$, respectively, in 20 mM cacodylate buffer (pH 7.4) at 2°C .

chromes in separate tandem cells (spectrum B). These two spectra presumably represented

$$[(c_1^{2+} \cdot c^{2+}) - (c_1^{2+} + c^{2+})] \text{ and } [(c_1^{3+} \cdot c^{3+}) - (c_1^{3+} + c^{3+})],$$

with distinct minima at 416 and 407 nm (± 1 nm), respectively.[‡] Recorded spectra differed from those of these difference spectra, depending on the reduction state of the two cytochromes.

When the difference spectrum obtained 12 msec after mixing c_1^{2+} with c^{3+} (Fig. 1B) were compared with spectrum A in Fig. 2, no isosbestic point was found. This phenomenon was observed if, before mixing, the c_1^{2+} concentration was higher or equal to the c^{3+} concentration.

In the course of reaction between c_1^{2+} and c^{3+} (Fig. 3A), when the concentration of c_1^{2+} was lower than that of c^{3+} , another spectral result was obtained after mixing, producing an absorbance increase at 408 ± 1 nm (Fig. 3B) with a small absorbance decrease in the 416-nm region. In this case, the difference spectrum obtained 12 msec after mixing could be compared with the difference spectrum of $[(c_1^{3+} \cdot c^{3+}) - (c_1^{3+} + c^{3+})]$ (Fig. 2, spectrum B). The spectral variations during electron transfer between these two cytochromes did not accord with the difference spectrum of $[(c_1^{2+} + c^{3+}) - (c_1^{3+} + c^{2+})]$ expected from the spectra obtained in the equilibrium study.

The k_{obs} at either 417 or 408 nm were found to be equal to $48 \pm 9 \text{ sec}^{-1}$ (Fig. 4) but did not agree with values for any of the following constants: $k(c_1^{2+})$, $k(c^{3+})$, $k[(c_1^{2+}) + (c^{3+})]$ or $k(c_1^{2+})(c^{3+})$. The amplitude of the difference spectra depended on c_1 concentration (at least from 0.75 to $5 \mu\text{M}$) as shown in Fig. 4. However, the amplitude of the spectra and the rate constants were found to be independent of the ionic strength of cacodylate buffer used from 0.02 to 0.5 M.

For the back-reaction (c_1^{3+} with c^{2+}), the phenomena ob-

FIG. 2. Difference spectra of the cytochrome complexes. Spectrum A, $(c_1^{2+} \cdot c^{2+}) - (c_1^{2+} + c^{2+})$; spectrum B, $(c_1^{3+} \cdot c^{3+}) - (c_1^{3+} + c^{3+})$. Spectra were traced at 2°C in 20 mM cacodylate buffer. Tandem cuvettes were used. Overall light path was 0.875 cm. Spectrum A was obtained using $3.45 \mu\text{M}$ each c_1^{2+} and c^{2+} . Spectrum B was obtained using $2.55 \mu\text{M}$ each c_1^{3+} and c^{3+} .

served in the forward reaction were reversed; that is, when the concentration of c_1^{3+} was higher than that of c^{2+} , the absorbance at 408 ± 1 nm decreased as a function of time, corresponding presumably to the formation of the ferricytochrome complex ($c_1^{3+} \cdot c^{3+}$), and when the concentration of c_1^{3+} was lower than that of c^{2+} , only the increase of absorbance at 416 ± 1 nm was observed, corresponding to the formation supposedly of the complex ($c_1^{2+} \cdot c^{2+}$). The rate constant, k_{obs} , was about 45% higher for the back-reaction as compared with that for the forward reaction. It decreased slightly, not more than 20%, with increasing ionic strength.

To obtain more information, the temperature effect on the

FIG. 3. Electron transfer between c_1^{2+} and c^{3+} . (A) Kinetic tracing at absorption maximum (408 nm). (B) Rapid-scan spectrophotometric measurements of the reaction of c_1^{2+} with c^{3+} . From bottom to top, the spectra were obtained 12, 24, 48, and 96 msec after mixing—i.e., as in Fig. 1B except in reverse order. The spectrum number 4 is the base line (the flat spectrum) because the spectra were computer-calculated difference spectra against the reference of the final spectrum—i.e., 96 msec (cf. Fig. 1). Scanning time for each spectrum was 2 msec. All others are the same as in Fig. 1, except for the concentration of cytochromes ($0.75 \mu\text{M}$ for c_1^{2+} and $2.25 \mu\text{M}$ for c^{3+}).

[‡]In this paper, the c_1 - c complex, or more accurately the c_1 -hinge protein- c complex (c_1 -H- c complex), of the type as previously reported (3) requires a small protein (i.e., the hinge protein) for the complex formation (4). The complexes whose formation do not need the hinge protein are symbolized as the $c_1 \cdot c$ complex, to be distinguished from the stable c_1 -H- c complex.

FIG. 4. Effect of c_1 concentration of electron transfer between c_1 and c . Left-hand scale is the amplitude at 416 nm of the difference spectra, 12 msec after mixing, as a function of c_1 concentration. The concentration of ferricytochrome c is indicated in brackets on the curves. Right-hand scale is a plot of the k_{obs} in each reaction between c_1^{2+} and c^{3+} as a function of ferrocyclochrome c_1 concentration. Δ , k_{obs} values; +, change of average observed absorbance obtained from at least five determinations; — and ----, observed absorbance change and k_{obs} values as a function of c_1 concentrations, respectively.

rate of reactions was investigated. Kinetic experiments were performed while lowering the temperature step by step. We chose to decrease the temperature as the reaction between c_1 and c was very fast, even at 2°C. Ethylene glycol at 40% (vol/vol) was used to maintain the medium fluid when the temperature was decreased to -24°C (16). The pH, termed "p_{aH}" in hydroorganic media, was adjusted accordingly to 7.4 in the temperature range used (10, 11). Using the concentrations of c_1^{2+} and c^{3+} , 2.5 μM and 0.5 μM, respectively, the rate constant, k_{obs} , obtained at 2°C, in the presence of 40% (vol/vol) ethylene glycol was higher than in pure aqueous buffer of 20 mM cacodylate (pH 7.4); it was 120 and 48 sec⁻¹, in the presence and absence of ethylene glycol, respectively. When the temperature was lowered, it decreased—i.e., at -5°C, k_{obs} was 90 sec⁻¹.

At both 2°C and -5°C, the same characteristic spectral variations could be observed at 416 nm as in pure aqueous buffer (20 mM cacodylate, pH 7.4). However, when the temperature was lower than -20°C, the spectral variations at 416 nm were not observed; instead, the spectral characteristics with maximum at 408 nm appeared (Fig. 5A). At -10°C, practically no signal was evident (Fig. 5B). These observations may have been consequent on a variation of equilibrium between the two complexes as in $(c_1^{2+} \cdot c^{3+}) \rightleftharpoons (c_1^{3+} \cdot c^{2+})$ through possible intermediates. No accurate activation energy for this process can be estimated. Between 2°C and -5°C, it is only possible to guess it to be 6 ± 2 kcal·M⁻¹ (1 cal = 4.184 J).

These results suggest that at least two intermediates are formed in the course of electron transfer between c_1 and c , according to the following mechanism:

where K_1 and K_3 symbolize rapid equilibria with association constants for the first and last steps $\gg 10^6$ M⁻¹.

The measured constant (k_{obs}) does not depend on the concentration of c_1 , but the amplitude is proportional to it (Fig. 4). We consider that for the direct reaction when concentrations $[c_1^{2+}] \leq [c^{3+}]$, all c_1 is completely complexed with (or saturated by) c^{3+} with only an excess of c^{3+} free, and that for the reverse reaction c^{2+} is completely complexed with c_1^{3+} .

FIG. 5. Rapid-scan spectrophotometric measurements of the reaction of ferrocyclochrome c_1 with ferricytochrome c as a function of temperature. Ferrocyclochrome c_1 and ferricytochrome c concentrations are 2.5 and 0.5 μM, respectively. The medium consists of cacodylate buffer containing 40% (vol/vol) ethylene glycol (p_{aH}, 7.4). Ionic strength is 20 mM. Numbers on the figure indicate the elapsed time after mixing, in msec. Scanning time was 2 msec for each spectrum. A, B, and C are at -5.2°C, -10°C, and -15°C, respectively. The bottom line in A is 96 msec, and the top line in C is 96 msec.

The two rate constants (k_{obs}) for the direct and forward reactions are the same within experimental error.

Kinetically, there is a rather simple reaction—a unimolecular reversible process as in Eq. 2.

with the equilibrium constant $K = k_2/k_{-2}$.

These results are quite different from those reported (5), in which a second-order rate constant of the forward reaction $c_1^{2+} + c^{3+} \rightleftharpoons c_1^{3+} + c^{2+}$ was determined that decreased from 3.0×10^7 M⁻¹ s⁻¹ at ionic strength 223 mM to 1.8×10^5 M⁻¹ s⁻¹ at 450 mM ionic strength at 10°C. Such a result could be rationalized by assuming that electron transfer between these two cytochromes occurred through an intermediate of the stable complex (c_1 -H- c) (3). However, the c_1 -H- c complex either in the oxidized, reduced, or mixed valence forms dissociated completely in media with ionic strength >100 mM (3).

Our evidence shows that the electron transfer reaction between cytochromes c_1 and c does not require the stable c_1 -H- c complex (3), which has been generally accepted as one of the characteristic interactions between these two cytochromes (see ref. 17). The complexes we must postulate—i.e., $c_1 \cdot c$ complex species, such as $c_1^{2+} \cdot c^{2+}$, $c_1^{3+} \cdot c^{3+}$, $c_1^{2+} \cdot c^{3+}$, and $c_1^{3+} \cdot c^{2+}$ —differ from the stable c_1 -H- c complex, which can be obtained only in the presence of the hinge protein (4, 8) in a medium of low ionic strength. Since they can be observed only by the spectral variation during the time in the course of low-temperature kinetic measurement and cannot be seen at equilibrium, it might be suggested that electron transfer between cytochromes c_1 and c requires some special conformational rearrangement. The role of the c_1 -H- c complex remains to be elaborated.

We may mention that we have characterized the hinge protein and determined its amino acid sequence. It has a total of 78 amino acid residues, with molecular weight 9175. The structure is unique in that near the NH₂-terminal eight

glutamic acid residues are linked consecutively (7). There is no spectral absorption at >300 nm, and even the absorption coefficient at the 280-nm region is very low because there is no tyrosine or tryptophan, but only two each of disulfide linkages and phenylalanine residues (8). The protein spectrum does not contribute to the visible absorption spectra of cytochrome c_1 or cytochrome c (unpublished data).

The question remains whether the complexes which appear at low temperatures are relevant to electron transfer at room temperatures.

The authors are grateful to Drs. T. Barman and F. Travers for their useful suggestions and stimulating discussion and to Professor P. Douzou for his interest and hospitality to C.H.K. and T.E.K. This work was supported by grants from the Delegation Generale a la Recherche Scientifique et Technique (Action Specifique, 81.7.04234), from the National Institutes of Health (GM-16767 and HLB-12576), and from the American Cancer Society (BC-349).

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