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Mutation-Induced Perturbation of the Cytochrome *c* Alkaline Transition†

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Received July 26, 1988; Revised Manuscript Received October 12, 1988

ABSTRACT: The possible influence of residue Phe-82 in the cytochrome *c* alkaline isomerization has been evaluated by spectrophotometric pH titrations of a family of mutant yeast iso-1-cytochromes *c* in which the identity of the residue at this position has been varied. The pK_a for the exchange of the Met-80 heme iron ligand was determined from pH titrations in which the S → Fe charge-transfer band (695 nm) was monitored and was found to be 8.5 for the wild type, 7.7 for Ser-82, 7.7 for Gly-82, 7.2 for Leu-82, and 7.2 for Ile-82. pH-jump experiments [Davis et al. (1974) *J. Biol. Chem. 249*, 2624] established that substitutions at position 82 affect the alkaline isomerization by lowering the pK_a of the titrating group by as much as 1.4 pK units; for the Ser-82 and Gly-82 variants, there is also a small effect on the K_{eq} for the ligand exchange equilibrium. On the basis of these findings, we conclude that one critical role for Phe-82 in the wild-type protein is stabilization of the native heme binding environment.

Residue Phe-82 of eukaryotic cytochromes *c* is phylogenetically conserved and has been implicated in the mechanism of electron transfer between cytochrome *c* and cytochrome *c* peroxidase (Poulos & Kraut, 1980). By use of the technique of site-directed mutagenesis, this residue has been replaced in yeast iso-1-cytochrome *c* by Ser, Tyr, Gly, Ile, or Leu (Pielak et al., 1985; Liang et al., 1988), and the resulting proteins have been found to demonstrate significant differences in their rates of electron transfer in photoexcited electron-transfer reactions with Zn-CCP (Liang et al., 1987, 1988). During subsequent studies we have discovered that mutations produced at position 82 unexpectedly destabilize the oxidized form of the cytochrome by lowering the pK_a for the conversion

†This research was supported by National Institutes of Health Grant GM-33804 to A.G.M. and by Medical Research Council of Canada Operating Grant MT-1706 to M.S. M.S. is a Career Investigator of the Medical Research Council of Canada.

0006-2960/89/0428-3152$01.50/0

of the native protein to the alkaline form. The present report describes the results of optical pH titrations and of pH-jump kinetic studies that we have undertaken with wild-type yeast iso-1-cytochrome c and several position 82 mutants to define the nature of this effect and gain some mechanistic understanding of its origin.

Experimental Procedures

Protein Preparation. Mutant forms of yeast iso-1-cytochrome c were generated and purified from *Saccharomyces cerevisiae* as described previously (Pielak et al., 1985; Cutler et al., 1987). In addition to the substitutions at position 82, all of the variants studied also possessed an additional substitution at position 102, in which the normally occurring cysteinyl residue was replaced with a threonyl residue. This substitution prevents dimerization through intermolecular disulfide bond formation (Narita & Titani, 1969) and reduces the rate of ferricytochrome c autoreduction to the level observed for horse heart ferricytochrome c (Cutler et al., 1987). The wild-type form of yeast iso-1-cytochrome c with Cys-102 was obtained commercially (Sigma type VIII) and purified by ion-exchange chromatography by the same procedure employed for the recombinant proteins.

pH Titrations. Ferricytochrome c solutions (0.2 mM) were exchanged into 0.1 M NaCl by overnight dialysis, and the pH of the solutions was then titrated to a value 2 pH units below the anticipated pK for the alkaline transition of the protein under consideration with either 0.1 M HCl or 0.1 M NaOH. For the Cys-102-containing wild-type protein, $NH_3[Co(dipic)_2]$ (0.2 mM) (Mauk et al., 1979) was added to the solution to prevent autoreduction. The optical/pH titrations were performed by transferring the protein solution into a modified quartz cuvette (1-cm path length) that was extended at the top to accommodate several milliliters of solution which contained a small magnetic stirring bar. This cuvette was then mounted in the cell holder of a Cary 219 spectrophotometer equipped with a magnetic stirrer accessory. The cell holder was maintained at 25 °C with a circulating water bath. The pH of the protein solution was continuously monitored with an in-dwelling combination microelectrode (Model MI-412; Microelectronics, Londonderry, NH) connected to a Radiometer Model 84 pH meter. The pH of the solution was raised incrementally by addition of 0.1 M NaOH (with stirring) in small volumes from a Manostat digital buret. In general, less than 30 μL of base was required to raise the pH of 3 mL of protein solution to a value of 11. After addition of NaOH solution, the absorbance at 695 nm was monitored until it reached a stable value, and the spectrum from 600 to 800 nm was then scanned.

pH-Jump Kinetics. The kinetics of the pH-induced conformational change of cytochrome c was studied by a pH-jump technique originally described by Davis et al. (1974). In these experiments, ferricytochrome c (40 μM) was exchanged into 0.1 M NaCl by dialysis. For experiments in which the pH of the cytochrome solution was increased rapidly, the pH of the protein solution was adjusted to a value that was 2 pH units lower than the pK for the transition measured for the protein in the pH titration experiments. For experiments in which the pH of the cytochrome solution was decreased rapidly, the initial pH of the protein solution was adjusted to a value 1 pH unit greater than the pK of the transition as measured above.

The pH-jump experiments were performed by rapidly mixing these protein solutions with sodium phosphate or sodium borate–NaCl buffers (μ = 0.1 M) with pH values ranging from 5.5 to 10 and monitoring the rate of change in absorbance at 695 nm. Rapid mixing was accomplished through use of a Dionex stopped-flow spectrophotometer interfaced to a Zenith Model Z-100 microcomputer (On-Line-Instrument-Systems, Jefferson, GA). Typically, absorbance changes of 0.01 to 0.02 OD unit were observed.

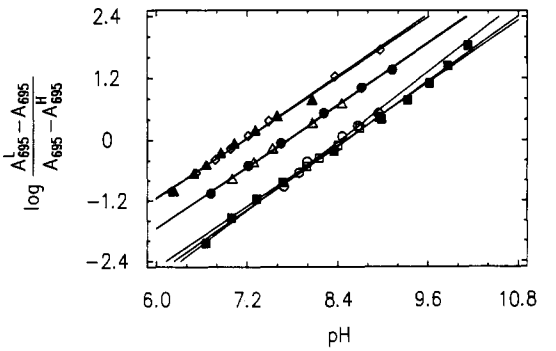


FIGURE 1: pH titrations of wild-type and mutant yeast iso-1-cytochromes c. The absorbance was monitored at 695 nm as a function of pH (25 °C, 0.1 M NaCl, [cytochrome c] = 0.2 mM) and analyzed by a linear least-squares fit to the data. A^H is the absorbance of the alkaline form of the protein at 695 nm, and A^L is the absorbance of the native form. The slopes are approximately 1, indicating that one proton is involved in the conformational change. (□) Cys-102 wild type; (■) Thr-102 wild type; (○) Tyr-82 mutant; (●) Ser-82 mutant; (△) Gly-82 mutant; (▲) Ile-82 mutant; (◇) Leu-82 mutant.

Table I: pK_a Values for Alkaline Transition of Cytochromes c Determined from Titrations[a]

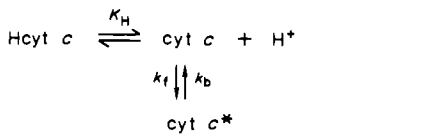
protein	pK_a	protein	pK_a
Cys-102	8.5	Gly-82/Thr-102	7.7
Thr-102	8.5	Ile-82/Thr-102	7.2
Tyr-82/Thr-102	8.4	Leu-82/Thr-102	7.2
Ser-82/Thr-102	7.7	horse heart	9.1

[a] Conditions: [cytochrome c] = 0.2 mM, μ = 0.1 M (NaCl), 25 °C.

Results

pH Titrations. Analysis of the change in absorbance at 695 nm for the wild-type and mutant cytochromes as a function of pH was based on the assumption of two pH-dependent forms of the ferricytochrome. Accordingly, the apparent pK for the equilibrium between the two forms was determined for each protein from a linear least-squares fit to the data plotted as log $[(A^L_{695} - A_{695})/(A_{695} - A^H_{695})]$ vs pH, where A^H_{695} is the absorbance of the alkaline form of the protein at 695 nm and A^L_{695} is the absorbance of the native form of the protein at 695 nm. The results obtained for each of the cytochromes are shown in Figure 1. The slopes determined for each of these titrations are approximately 1, which indicates that one proton is involved in the conformational change observed. The pK values determined from the data shown in Figure 1 are summarized in Table I. While the Cys-102 wild-type protein, the Thr-102 variant, and the Tyr-82/Thr-102 variant are found to have similar values for this pK, the other variants differ substantially in their behavior. We note that these other proteins fall into two categories: the Gly-82/Thr-102 and Ser-82/Thr-102 mutants with pK values of 7.7 and the Ile-82/Thr-102 and Leu-82/Thr-102 mutants with pK values of 7.2.

pH-Jump Kinetics. From the analysis developed by Davis et al. (1974), pH-jump kinetics of the type performed here can be described adequately by the mechanistic scheme:



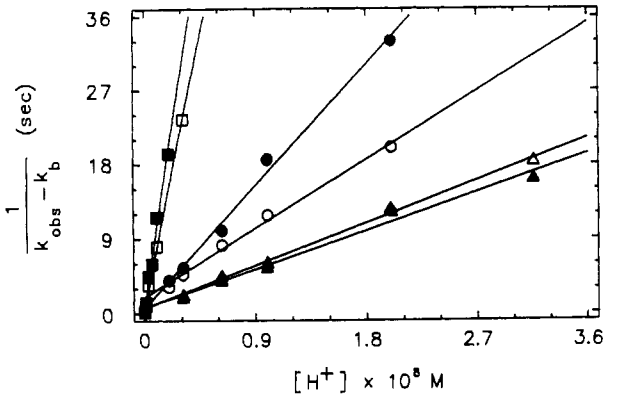


FIGURE 2: pH-jump kinetics of wild-type and mutant cytochromes (25 °C, μ = 0.1 M, [cytochrome *c*] = 40 μM). The solid lines represent linear least-squares analyses of $1/(k_{obsd} - k_b)$ vs $[H^+]$ according to eq 4. The rate of change was monitored at 695 nm. (□) Cys-102 wild type; (■) Thr-102 wild type; (○) Gly-82 mutant; (●) Ser-82 mutant; (▲) Ile-82 mutant; (△) Leu-82 mutant.

where native cytochrome *c*, possessing the maximum at 695 nm, is denoted as Hcyt *c* or cyt *c* and the alkaline form of the protein is denoted as cyt *c**. This mechanism consists of an initial deprotonation step described by pK_H and a subsequent conformational equilibrium between the deprotonated native conformation and the alkaline form of the protein that is described by the equilibrium constant K_C ($=k_f/k_b$).

The kinetic equations that are relevant to the mechanism described above are (Davis et al., 1974)

$$\frac{dA_{695}}{dt} = -\frac{d([\text{Hcyt } c] + [\text{cyt } c])}{k_b(K_H + [H^+]) + k_f K_H} = \frac{d[\text{cyt } c^*]}{dt} = k_f[\text{cyt } c] - k_b[\text{cyt } c^*] \quad (1)$$

$$[\text{cyt } c]_t = -\frac{-k_f K_H C_T}{k_b(K_H + [H^+]) + k_f K_H}(e^{-k_{obsd}t-1}) \quad (2)$$

$$k_{obsd} = k_b + k_f\frac{K_H}{K_H + [H^+]} \quad (3)$$

Derivation of these relationships assumes that the deprotonation equilibrium is rapid with respect to the conformational isomerization equilibrium. In these equations, C_T represents the total concentration of cytochrome *c*.

In the present work, the values of k_f and k_b were calculated from a linear rearrangement of eq 3:

$$\frac{1}{k_{obsd} - k_b} = \frac{1}{k_f} + \frac{1}{k_f K_H}[H^+] \quad (4)$$

The values of k_b were determined experimentally by a pH-jump experiment involving a rapid decrease in pH. As can be seen from eq 3, when $[H^+] \gg K_H$, k_{obsd} is approximately equal to k_b.

The linear least-squares analysis of the $1/(k_{obsd} - k_b)$ vs $[H^+]$ plots based on eq 4 for each of the mutants is shown in Figure 2. Values of k_f were obtained from the intercepts and of K_H were obtained from the slopes of these lines for each case and are set out in Table II. K_C values were calculated from the ratio of the forward and reverse rate constants for this equilibrium (k_f/k_b). The value of the p*K* for the alkaline isomerization calculated from these parameters ($pK_C + pK_H$) are also included in Table II for comparison with the values obtained from the pH titrations discussed above. Although as outined by Davis et al. (1974), the intrinsic error in determination of pK_C is relatively large, the pK_a for the alkaline transition obtained from the two types of experiment are within experimental error of each other.

Table II: Kinetic Parameters for Alkaline Transition of Mutant Yeast Iso-1-cytochromes *c* from pH-Jump Experiments[a]

protein	$k_b \times 10^2$ (s^{-1})	k_f (s^{-1})	K_C	pK_H	$pK_H + pK_C$
Cys-102 w.t.	3.5 (1)	8.5 (3)	244 (11)	11.0 (1)	8.6 (2)
Thr-102	2.9 (4)	13.4 (7)	460 (40)	10.9 (1)	8.3 (3)
Gly-82/Thr-102	4.9 (4)	1.1 (2)	22 (3)	9.1 (3)	7.8 (6)
Ser-82/Thr-102	5.5 (5)	0.9 (2)	16 (5)	9.2 (7)	8 (1)
Ile-82/Thr-102	2.2 (2)	4 (1)	200 (40)	9.4 (6)	7 (1)
Leu-82/Thr-102	3.3 (4)	7 (2)	210 (90)	9.6 (7)	7 (1)

[a] The standard deviations of the values cited are indicated in parentheses.

Discussion

The occurrence of five pH-dependent forms of cytochrome *c* was first reported nearly 50 years ago by Theorell and Åkesson (1941). Since that time, most effort has been directed at understanding the structural and functional properties of those forms of the protein that exist at neutral pH (the native form) and at alkaline pH (the alkaline form). The majority of the effort to understand the alkaline form of the protein has generally involved either spectroscopic and structural analysis of the alkaline form of the protein to define the nature of the axial ligands to the heme iron or kinetic studies to determine the mechanistic nature of the conversion of the native form of the protein to the alkaline form. Despite the extensive effort that has been expended toward identification of the axial ligands in the alkaline form of the protein, the resulting conclusions are not entirely consistent [see references reviewed in Brautigan et al. (1977) and in Gadsby et al. (1987)]. There is little disagreement, however, that at high pH the Met-80 ligand to the iron in the native protein is replaced by some other amino acid residue. The most frequent suggestion concerning the identity of the new ligand at high pH is that it is an ϵ-amino group from a lysyl residue, a substitution that would require a major conformational change in the protein.

Those mechanistic studies directed at understanding the native–alkaline transition have involved pH titrations and pH-jump kinetic techniques similar to those employed in the present work, though (as with the spectroscopic studies) horse heart cytochrome *c* has been the species of protein used most commonly. The results of pH-titration experiments for both horse heart cytochrome *c* and yeast iso-1-cytochrome *c* as monitored by a variety of spectroscopic techniques are summarized in Table III. Despite the scatter in the reported results, it is clear that the p*K* for this transition is different for the two species, with the value for the horse heart protein being approximately 0.5 pK unit greater than that for the yeast cytochrome. The structural basis for this difference is currently unknown.

The present observation that substitutions at a single residue can produce significant alterations in the pH-dependent behavior of cytochrome *c* suggests that analysis of specifically mutated cytochromes will provide considerable insight into the mechanistic origin of the alkaline transition. Both the titration data and the kinetic data indicate that the four cytochrome variants demonstrating a modification in the alkaline transition fall into two classes. One category is comprised of the Ser-82 and Gly-82 variants, in which the pK_a is reduced approximately

Table III: Values of the Alkaline Transition pK_a for Horse Heart and Yeast Cytochrome c Reported in the Literature

technique	conditions	pK_a	ref
	Horse Heart Cytochrome c		
electronic spectroscopy	0.1 M NaCl, 0.1 mM protein, 25 °C	9.1	this work
electronic spectroscopy	60 mM phosphate, 50–90 μM protein, temperature not stated	9.5	*a*
electronic spectroscopy	25 mM sodium borate, 0.3 M NaCl, 1 mM protein, 22 °C	9.3	*b*
electronic spectroscopy	μ = 0.1, 0.2 mM protein, 21 °C	9.0	*c*
electronic spectroscopy	50 mM Tris, 0.1 or 0.5 mM protein, temperature not stated	9.4	*d*
electronic spectroscopy	μ = 0.02 M, 0.5 mM protein, 25 °C	9.2	*e*
electronic spectroscopy	μ = 0.3 M, 0.5 mM protein, 25 °C	8.9	*e*
electronic spectroscopy	10 mM Tris/acetate, [protein] not stated, 25 °C	9.05	*f*
electronic spectroscopy	50 mM KCl or 50 mM borate–50 mM KCl, 7–50 μM protein, temperature not stated	8.9	*g*
^{1}H NMR spectroscopy	unstated	~9	*h*
^{1}H NMR spectroscopy	0.1 M NaCl, 6 mM protein, 24 °C	8.9	*i*
^{1}H NMR spectroscopy	no conditions stated, pH uncorrected for isotope effect	9.0	*j*
^{13}C NMR spectroscopy	0.2 M NaCl, 5 mM protein, 20 °C	9.1	*i*
EPR spectroscopy	water, 1 mM protein, 2 K	9.7	*a*
EPR spectroscopy	25 mM sodium borate, 0.3 M NaCl, 1 mM protein, 21 K	9.3	*b*
MCD spectroscopy	50 mM CAPS, 50 mM CHES, 50 mM TAPS, room temperature, [protein] not stated	9.3	*k*
pH-jump kinetics	μ = 0.1/0.2 M, NaCl, 40 mM protein, 25 °C	9.0	*c*
reduction kinetics	25 mM sodium borate, 0.3 M NaCl, 1 mM protein, 25 °C	9.3	*b*
resonance Raman spectroscopy	0.7 mM protein, no buffer or temperature stated	9.4	*l*
	Yeast Iso-1-cytochrome c		
electronic spectroscopy	0.1 M NaCl, 0.1 mM protein, 25 °C	8.5	this work
electronic spectroscopy	60 mM phosphate, 50–90 μM protein, temperature not stated	8.6	*a*
electronic spectroscopy	50 mM Tris, 0.1 or 0.5 mM protein, 20 °C	8.41	*d*
CD spectroscopy	50 mM Tris, 20 °C, [protein] not stated	8.35	*d*
EPR spectroscopy	0.1 M phosphate (pH 2–9), 0.2 M bicarbonate (pH 9.5 and 10.2), 1 M ammonia (pH 11), 1 N NaOH (pH 13), 1–3 mM protein, 4.2 K	8.6	*a*

[a] Brautigan et al., 1977. [b] Lambeth et al., 1973. [c] Davis et al., 1974. [d] Looze et al., 1978. [e] Greenwood & Wilson, 1971. [f] Osheroff et al., 1980. [g] Smith & Millett, 1980. [h] Gupta & Koenig, 1971. [i] Wooten et al., 1981. [j] Falk et al., 1981. [k] Gadsby et al., 1987. [l] Kitagawa et al., 1977.

0.8 unit to 7.7. As determined from the pH-jump experiments, the reduction of this pK_a for these two proteins arises from a change in the pK_a of the (as yet unidentified) titrating group that appears to be linked to the alkaline transition and a change in the equilibrium constant for the conformational equilibrium between the native and alkaline forms of the protein. The other category is comprised of the Leu-82 and Ile-82 variants, in which the pK_a is reduced by approximately 1.3 pK units to 7.2. In this case, the pH-jump experiments demonstrate that the sole effect of the mutations is a change in the pK_a of the titrating group that is linked to the transition. The identity of this group and that of the residue which provides the sixth ligand to the iron atom in the alkaline form remain indeterminant. We note, however, that the current results appear to argue against the suggestion of Gadsby et al. (1987) that the former group is the proximal histidine residue. If the pK_a of this group were linked to the alkaline transition, that would mean that the mutations we have introduced at position 82 have the effect of lowering the abnormally low p*K* of this group 2 additional pK units (to a value of ca. 9). It seems more reasonable that destabilizing mutations should, if anything, cause a pK_a that is aberrant in the wild-type protein to approach a more normal value.

The perturbation of the alkaline transition resulting from substitutions at position-82 raises the possibility that our previous observations concerning the effect of these mutations on the rate of electron transfer from ferrocytochrome *c* to the Zn–cytochrome *c* peroxidase porphyrin π-cation radical may have been unknowingly affected by the effect of the mutations on this equilibrium. Two facts argue against this possibility. First, the effect of pH on the coordination of the heme iron is generally acknowledged to involve ferricytochrome *c* only; the integrity of the methionine–iron bond of ferrocytochrome *c* derivatives at alkaline pH is clear. As the most profound kinetic effects of mutations at position-82 we have observed involve the reduced form of the cytochrome, the effect of the mutations on the alkaline isomerization should not influence our kinetic findings. Second, the rate of electron transfer from the reduced Ser-82 mutant to Zn–cytochrome *c* peroxidase porphyrin π-cation radical is the same at pH 6 as previously observed at pH 7 (N. Liang and B. M. Hoffman, unpublished results). If the decrease in alkaline p*K* were in some way affecting our kinetic observations, then we would have expected that the rates observed at the two values of pH would differ.

We conclude from the present study that one role of Phe-82 is the stabilization of the heme binding domain of cytochrome *c* by preventing formation of the alkaline form of the protein near physiological values of pH. This observation is in concert with suggestions made by Osheroff et al. (1980) on the basis of pH titrations of several cytochrome *c* species. From their comparative studies, these authors speculated that Phe-82, Ile-9, Ile-85, and Leu-94 constitute a hydrophobic domain at one end of the heme crevice that appears to stabilize the heme environment against transition to the alkaline form. The current results suggest that hydrophobicity alone is not a sufficient criterion to achieve this stabilization inasmuch as the Leu-82 and Ile-82 variants exhibit a considerable decrease in the observed values for their alkaline transition pK_a. Finally, we note that the current family of mutants was originally prepared to study the role of residue 82 in the mechanism of cytochrome *c* electron transfer and that the perturbation in the alkaline isomerization process observed here arose as an unanticipated consequence of mutagenesis. This occurrence demonstrates the necessity of thorough characterization of any mutant proteins that are generated by protein engineering to assure that the properties of initial interest in the mutagenic experiment are not the only properties to be affected by the modifications introduced.

ACKNOWLEDGMENTS

We thank Dr. Geoffrey R. Moore and Prof. Gary D. Brayer for helpful discussions and suggestions. We also thank Professor Brian M. Hoffman for communicating results concerning the effect of pH on the rate of reaction between the

Ser-82 mutant and Zn-substituted cytochrome *c* peroxidase prior to publication.

Registry No. L-Phe, 63-91-2; L-Ser, 56-45-1; L-Gly, 56-40-6; L-Leu, 61-90-5; L-Ile, 73-32-5; cytochrome *c*, 9007-43-6.

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Intrinsic Uncoupling in Proton-Pumping Cytochrome *c* Oxidase: pH Dependence of Cytochrome *c* Oxidation in Coupled and Uncoupled Phospholipid Vesicles†

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Received October 11, 1988; Revised Manuscript Received December 22, 1988

ABSTRACT: The pH dependence of the transient aerobic kinetics of cytochromes *c* and *a* has been investigated with cytochrome oxidase reconstituted in phospholipid vesicles in the absence and presence of an uncoupler and an ionophore. The cytochrome *a* reduction level immediately after the burst phase was 60-80% and was not significantly changed by the addition of uncoupler and/or ionophore. The coupled rate of ferrocytochrome *c* oxidation increases linearly with decreasing pH in the range 8.4-5.4. The increase in rate on uncoupling becomes less with decreasing pH and low cytochrome *c* concentration, being almost zero at pH 5.4. The coupled rate is increased by a lowering of the outside pH when the inside pH is constant. Varying the inside pH with a constant outside pH of 7.4 has little effect on the rate. It is suggested that the electrochemical potential has two separate effects on the coupled rate: the pH gradient mainly slows down the intramolecular electron transfer, but the membrane potential also lowers the second-order rate constant for the reaction with cytochrome *c*. The results are interpreted in terms of a model in which protonation of an acid-base group with a pK_a of 6.4 from the inside increases the catalytic constant. Protonation from the outside, on the other hand, leads to an intrinsic uncoupling, because the protonated enzyme in the output state can return to the input state. This has no adverse physiological effect, since it becomes significant only at pH values well below 7.

Cytochrome *c* oxidase, the terminal enzyme in cellular respiration, is a redox-linked proton pump (Wikström et al., 1981). There are certain basic principles governing the operation of such pumps, as emphasized in several recent reviews (Malmström, 1985; Blair et al., 1986; Krab & Wikström, 1987). An analysis of the kinetic properties of the enzyme within the framework of these principles had led to the formulation of a detailed reaction cycle, which describes the coupling between the catalytic electron-transfer reaction and the proton translocation (Malmström, 1987; Brzezinski & Malmström, 1987; Thörnström et al., 1988). According to this model the enzyme exists in two conformations, E_1 and E_2. Cytochrome *c* donates electrons to the primary acceptors, cytochrome *a* and Cu_A, in the E_1 conformation. The intramolecular electron transfer from the primary acceptors to the binuclear cytochrome a_3–Cu_B site, and the subsequent reaction

† This work was supported by grants from the Swedish Natural Science Research Council.

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0006-2960/89/0428-3156$01.50/0