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Proton Titration Curve of Yeast Iso-1-ferricytochrome *c*. Electrostatic and Conformational Effects of Point Mutations†

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ABSTRACT: The proton titration curves of yeast iso-1-ferricytochrome *c* and selected point mutants of this protein have been determined between pH 3 and 11 at 10 and 25 °C with a computer-controlled titration system. Initial titration of the wild-type protein to acidic pH followed by subsequent titrations to alkaline and then acidic pH demonstrates hysteresis, with one more group (28.7) titrating between pH 11 and 3 than originally titrated (27.7) between pH 3 and 11. Initial titration to alkaline pH, however, resulted in observation of the same number of groups in both directions of titration (28.7 vs 28.6). At 10 °C, 7.5 fewer groups were found to titrate over the same range of pH. Titration curves obtained for six cytochrome *c* mutants modified at Arg-38, Phe-82, Tyr-48, and Tyr-67 were analyzed by subtraction of the corresponding titration curve for the wild-type protein to produce difference titration curves. In most cases, the effects of these mutations as revealed in the difference titration curves could be accounted for as either the result of introduction of an additional group titrating within this pH range, the result of a change in the pK of a titrating residue, and/or the result of a change in the pK for either the first acidic or the first alkaline protein conformational transition. In addition to demonstration of the electrostatic consequences of the mutations in cytochrome *c* studied here, this study establishes the general usefulness of precise proton titration curve analysis in the characterization of variant proteins produced through recombinant genetic techniques.

The proton titration curve of a protein represents the number of equivalents of hydrogen ions bound or released in response to changes in pH. Consequently, such curves can be regarded as the summed contributions of the titratable groups in a particular protein and the manner in which these groups are influenced by the electrostatic characteristics of their environments (Linderstrøm-Lang, 1924; Tanford, 1962; Nozaki & Tanford, 1967). Unequivocal assignment of individual transitions observed in protein titration curves to specific titratable groups is, however, virtually impossible without additional information. If the ionization state of a particular group can be observed by another technique [e.g., ^{1}H NMR pH titrations (e.g., Meadows, 1972) or UV spectrophotometric pH titrations of tyrosyl residues], then the pK_a of such a group can be assigned unambiguously. Alternatively, a method for simulating the entire titration curve for those instances where the atomic coordinates of the protein are available from crystallographic analysis has been described in detail (Matthew et al., 1985; Matthew, 1986; Matthew & Gurd, 1986). Despite the approximations that are employed in such simulations concerning the nature of the dielectric within the protein and at the protein surface and therefore of the electrostatic in-

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teraction between titratable groups, the resulting simulated titration curves have proved to be remarkably similar to those determined experimentally.

A few reports have suggested alternatives to analysis of absolute titration curves in attempts to simplify interpretation of such data. For example, van Os and co-workers demonstrated the usefulness of differential titration curves in the analysis of the titration behavior of hemoglobins from several species (De Bruin & van Os, 1968; Janssen et al., 1970, 1972). Godinho et al. (1981, 1982) have applied modified Gran functions to linearize the titration curves of ovalbumin and lysozyme in segments as a means of identifying classes and populations of titrating residues. The effect of conformational change or chemical modification on protein titration behavior has been analyzed by using potentiometric difference titrations (Rossi-Bernardi & Roughton, 1967; Perlman, 1972; Parsons & Rafftery, 1972a,b). In the present study, we have used digitally acquired titration curves to calculate difference titration curves as a means of comparing the behavior of proteins having nearly identical structural properties.

Electrostatic interactions are critically important to the functional properties of small, soluble electron-transfer proteins such as the eukaryotic cytochromes *c*. For example, the reduction potentials of such proteins are, in part, a function of the electrostatic environment of the prosthetic group (Moore et al., 1986). In addition, the interaction of cytochromes *c* and electron-transfer proteins with which they react physiologically involves a number of electrostatic interactions [reviewed by Pettigrew and Moore (1987)]. A great deal of such information has been derived from the study of mammalian cytochromes *c*, yet relatively little of the pH-dependent behavior of this protein has been assigned to the titration of particular groups.

Despite the sensitivity of proton titration curves of proteins, the substantial information content of protein titration curves, and the extensive use of this approach in the characterization of proteins reported in the literature, application of this analytical technique to the study of recombinant proteins and mutants derived from them does not appear to have been reported previously. We now demonstrate that comparison of mutant proteins with corresponding wild-type proteins through determination of mutation-induced difference titration curves can in many cases provide detailed insight into the effects of the mutations on the electrostatic and conformational stability properties of the protein.

Experimental Procedures

Wild-type yeast iso-1-cytochrome *c* and the mutants His-38, Ala-38, Ser-82, Ile-82, Phe-48, and Phe-67 were isolated as described previously (Cutler et al., 1987; Pearce et al., 1989; Rafferty et al., 1990). All forms of yeast iso-1-cytochrome *c* studied here also possessed the additional mutation of Thr-102 to eliminate intermolecular disulfide bond formation and greatly reduce the rate of autoreduction (Cutler et al., 1987). Samples of FPLC-purified proteins were exchanged into water by ultrafiltration in a Centriprep-10 (Amicon). Approximately 3.5 mg of cytochrome *c* was then deionized by passage over 0.4 mL of MB-3 mixed-bed ion-exchange resin (Sigma, A-7518) and collected into a sealed bottle under an argon atmosphere. The concentration of the ferricytochrome solution was determined from its absorbance at 409.5 nm [ϵ = 106 100 M^{-1} cm^{-1} (Margoliash & Frohwirt, 1959)] at pH 7.2 or, for the Ser-82 mutant, at pH 6.0. The presence of some alkaline form in the Ile-82 mutant even at pH 6.0 (Pearce et al., 1989) required determination of its concentration at 410.6 nm (an isosbestic point for native and alkaline forms) with ϵ = 105 300 M^{-1} cm^{-1}. The Soret absorbance band maximum for Phe-67 was at 408.4 nm with ϵ = 107 400 M^{-1} cm^{-1} based on ϵ = 123 800 M^{-1} cm^{-1} at 406 nm for the alkaline form of both the Phe-67 mutant and the wild-type protein.

Approximately 2.5 mL (the exact volume for each titration was determined gravimetrically) of ferricytochrome *c* solution containing between 0.18 and 0.33 μmol of cytochrome *c* was transferred into a thermostated, sealed, glass titration vessel that was continuously flushed with humidified argon that had been passed through a CO_2-removing cartridge (Alltech 8119) and KOH pellets to remove CO_2. The pH of the protein solution was monitored until a stable value was reached ($\sim$25 min). This value was taken as the isoionic pH of the protein. The point of zero net charge was calculated by the method of Tanford (1962). Sodium chloride (1 M) was then added to achieve a final ionic strength of 0.1 M. The pH was again allowed to stabilize because the addition of NaCl caused an increase in the measured pH of approximately 0.2 pH unit. The pH was then adjusted to 5.5, and the sample was allowed to equilibrate for 30 min before titrations were started.

Titrations and pH measurements were performed with a Radiometer ABU93 Triburette that was equipped with three 1-mL burets and an SAM90 sample station housed within an aluminum Faraday cage. A glass electrode (Radiometer G222C) and a calomel reference electrode (Radiometer K401) were calibrated at the temperature of each experiment using standards at pH 4 (Radiometer S1316) and pH 10 (BDH RO1206-74). All measurements were made with maximal stirring speed. The titration assembly was interfaced to an IBM microcomputer for titrator operation and data acquisition. Automatic additions of variable volumes of titrant were made under software control [written in Turbo-C (Borland International)] at user-selected intervals (usually 120 s) while monitoring the pH of the solution. The pH was recorded as an average of four readings and stored with the added titrant volume before the next addition was made. The volume of titrant added was adjusted during the titration so that the incremental change in pH was 0.07 $\pm$ 0.01. The minimum volume of titrant added was 0.3 μL, and the maximum volume added was 6.0 μL. Titrants were CO_2-free 0.1 M HCl and 0.1 M NaOH which had been standardized against potassium acid phthalate (primary standard, Aldrich).

Digitized titration curves obtained in this manner consisted of 59–120 points each (each point comprising added volume vs pH). Each curve was fitted to a Chebychev polynomial (Horne & Parker, 1980) to obtain a curve of points, calculated from the polynomial coefficients, equally spaced along the pH axis. The fitted curve (20 points per pH unit) was converted to concentration of added titrant vs pH and then corrected by subtraction of the titrant concentration necessary to adjust an identical volume of 0.1 M NaCl to each corresponding pH value. The resulting corrected concentration of added titrant curve vs pH was converted to net proton charge vs pH. Spectrophotometric titrations were carried out at 25 °C on approximately 15 μM solutions of cytochrome *c* in 0.1 M NaCl using 0.1 M HCl as titrant. Data manipulation and titration curve simulations were performed with the program FITALL Ver. 4.0 (MTR Software, Toronto).

Electronic absorption spectra were obtained with a Cary 219 spectrophotometer fitted with a circulating water bath and interfaced to a Zenith Model Z-248 microcomputer (On-Line-Instrument Systems, Jefferson, GA).

Results

Wild-Type Cytochrome c. The proton titration curves for wild-type yeast iso-1-ferricytochrome *c* are shown in Figure

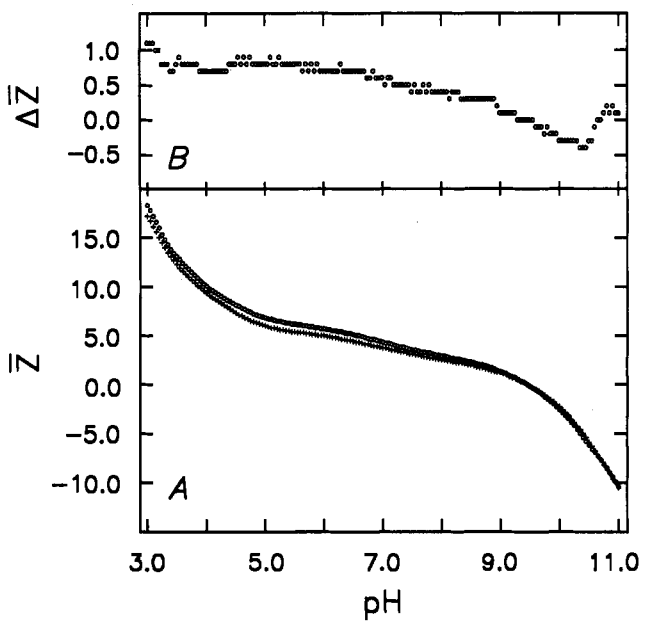

FIGURE 1: Potentiometric titration curves of wild-type yeast iso-1-cytochrome *c* in 0.1 M NaCl at 25 °C. $\bar{Z}$ is the net proton charge (Tanford, 1962); the pH of zero net charge was taken as 9.45. (A) Titration curve from pH 3 to 11 (+), collected first, followed by the titration from pH 11 to 3 (O). (B) The difference titration titration curve generated by subtraction of the upward titration (pH 3–11) from the downward titration (pH 11–3).

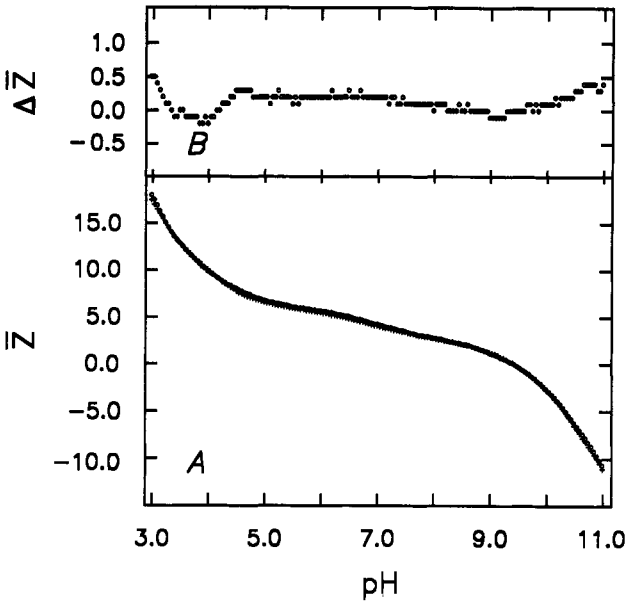

FIGURE 2: Potentiometric titration curves of wild-type yeast iso-1-cytochrome *c* in 0.1 M NaCl at 25 °C. (A) Titration from pH 11 to 3 (O), collected first, followed by titration from pH 3 to 11 (+). (B) The difference titration curve generated by subtraction of the upward titration (pH 3–11) from the downward titration (pH 11–3).

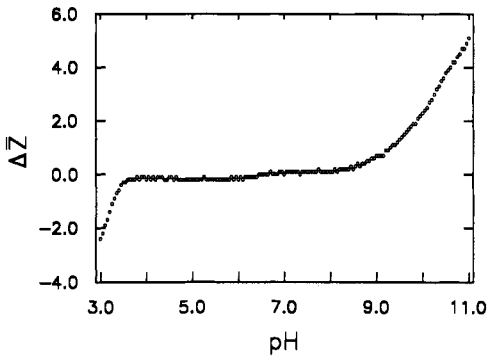

FIGURE 3: Effect of temperature on the potentiometric titration curve of wild-type yeast iso-1-cytochrome *c*. The titration curve collected at 25 °C was subtracted from that collected at 10 °C. Both titrations were recorded from pH 3 to 11 in 0.1 M NaCl. At 10 °C, the pH of zero net charge was taken as 9.90.

1. Titration of the wild-type protein between pH 3 and 11 exhibits a path-dependent behavior. When the protein was taken from its initial pH of 5.5 (the pH at which the samples were degassed by flushing with scrubbed argon) to pH 3.0 and then titrated from pH 3 to 11 and again from pH 11 to 3 (Figure 1A), an additional group was found to titrate on the final downward arm that was not seen in the upward direction (Figure 1B). Between pH 3 and 11, 27.7 groups titrated in the upward direction and 28.7 groups in the downward direction. This extra group titrated gradually and, therefore, is not readily assigned to a particular residue. However, if the protein was first taken from pH 5.5 to 11 and then titrated from pH 11 to 3, followed by titration from pH 3 to 11 (Figure 2A), the number of groups titrating over this range was the same in the downward direction (28.7 groups) as in the upward direction (28.6 groups) (Figure 2B). Separate titrations over different pH ranges suggest that the first exposure to a pH above 10 resulted in the generation of this additional titratable group. This appearance of an extra group was also observed with all the mutants examined. Note that the deionization of cytochrome *c* as required for potentiometric titrations unavoidably exposes the protein to conditions of moderately high pH. Therefore, to ensure reversibility, all subsequent titrations were performed from high to low pH and then vice versa.

The proton titration of wild-type cytochrome *c* at 25 °C was compared to that at 10 °C (Figure 3). Between pH 3 and 11, 7.5 fewer groups were titrated at the lower temperature. Five of these groups (Figure 3) titrate in the range pH 6–11 at 25 °C but not at 10 °C. We refer to the difference in number of titrating groups in this manner for convenience. It is important to note, however, that the differences seen at the acid and base extremes most likely represent the response of a population of partially titrated groups so that a change of "five groups" may result from smaller changes in the behavior of a larger number of groups. An increase in the p*K* for the alkaline isomerization of the protein with decreasing temperature would also contribute to the differences in charge of the protein at the two temperatures above pH 7.

The p*K* for the transition to a high-spin form of wild-type yeast iso-1-cytochrome *c*, assumed to be the equivalent of state II of horse heart cytochrome *c* (Dyson & Beattie, 1982), was determined to be 3.15 on the basis of spectrophotometric titration at 470 nm performed in 0.1 M Cl^-. This result is in good agreement with the report of Aviram and Schejter (1969). The corresponding transition for the horse heart protein occurs with a p*K* of 2.5 (Theorell & Åkesson, 1941; Dyson & Beattie, 1982). (Note: The uncertainty in pK_a values reported here for potentiometric or spectrophotometric titrations is ≤0.1.) At pH 1.59 (0.1 M Cl^-), the yeast protein exhibits a high-spin spectrum (Figure 4) with maxima at 395 nm (ϵ = 173 600 M^{-1} cm^{-1}), 496 nm (ϵ = 7560 M^{-1} cm^{-1}), and 619.5 nm (ϵ = 3420 M^{-1} cm^{-1}), similar to the high-spin acidic spectrum for horse heart cytochrome *c* (Aviram, 1973). These extinction coefficients are based on ϵ = 106 100 M^{-1} cm^{-1} for the native form of the protein. The slope of the plot of log ([high-spin acid form]/[native form]) vs pH (0.1 M Cl^-) yields n = 1.6 for the wild-type yeast protein.

Alanine-38 Mutant. Subtraction of the titration curve for the wild-type iso-1-cytochrome *c* from the titration curve for the Ala-38 mutant gives the difference titration curve shown

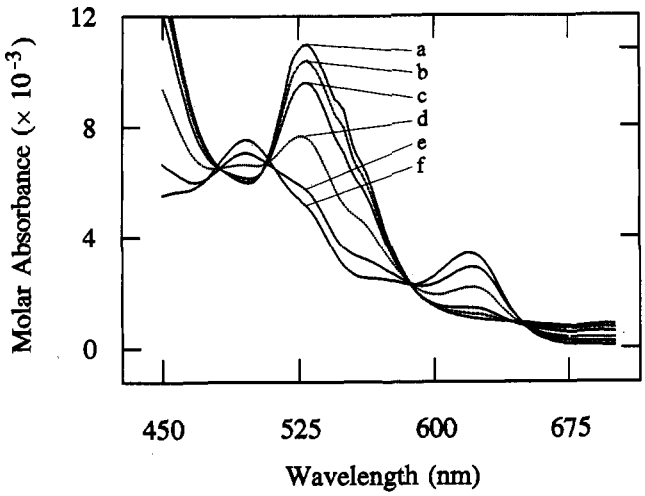

FIGURE 4: Electronic absorption spectrum of yeast iso-1-ferricytochrome *c* as a function of pH (25 °C, 0.1 M Cl^-) as follows: (a) 5.0; (b) 3.52; (c) 3.29; (d) 3.04; (e) 2.7; (f) 1.59.

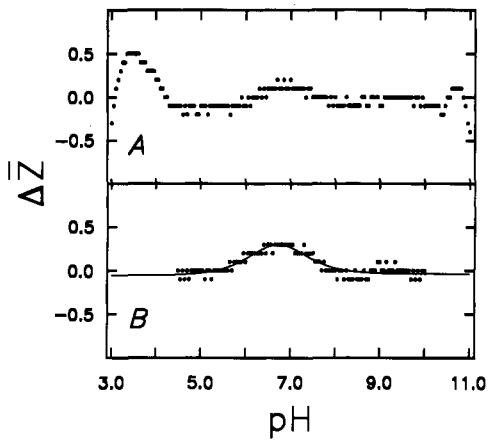

FIGURE 5: Difference titration curves of Ala-38 mutant minus wild-type cytochrome *c* at 25 °C in 0.1 M NaCl. (A) Difference between the titrations from pH 3 to 11. (B) Difference between the titrations from pH 10 to 4.5. The solid line represents the theoretical shift in the pK_a of a single ionizable group from 6.5 to 6.9.

in Figure 5A. The differences observed below pH 4.3 are attributed to a shift in the p*K* of the acid isomerization of the mutant relative to that of wild-type protein. This p*K* for the Ala-38 mutant was determined from spectrophotometric titrations at 525 and 470 nm to be 3.4 (n = 1.6). The origin of the perturbation in the Ala-38 difference titration curve observed at high pH (Figure 5A) has not been assigned. The shape of the difference curve near pH 7 is indicative of a shift in the pK_a of a single ionizable group upon mutation. Repeated titration between pH 4.5 and 10 indicated that this feature could be analyzed according to a shift in the pK_a from 6.5 in the wild-type protein to 6.9 in the Ala-38 mutant (Figure 5B). At 10 °C, the best theoretical fit to the difference titration curve in this region indicates a shift in the pK_a from 7.3 in the wild-type to 7.8 in the Ala-38 mutant (data not shown).

Histidine-38 Mutant. Figure 6A shows the difference titration curve at 25 °C of the His-38 mutant minus the titration curve of the wild-type cytochrome *c*. The total number of groups titrating between pH 3 and 11 in the His-38 mutant was found to be 29.7 compared to 28.6 in the wild-type protein. The most distinctive feature of this titration curve is the titration of an additional group with a pK_a of 8.2. Similar analysis of the difference titration curves obtained at 10 °C indicates that the pK_a of this new group increases to 8.8 at this lower temperature (Figure 6B). Significantly, the difference titration curve calculated for this mutant is consistent with the presence of a single additional titratable group in the mutant protein.

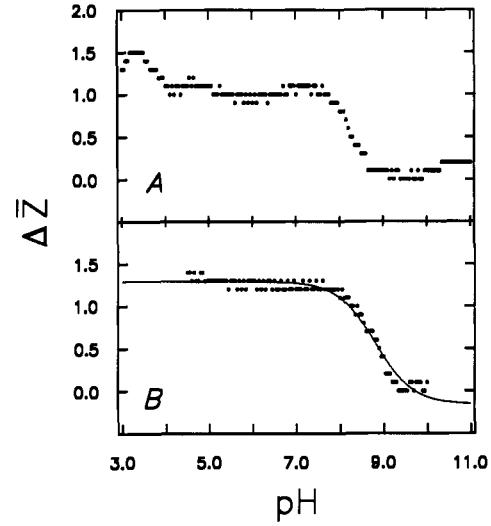

FIGURE 6: Difference titration curves of His-38 mutant minus wild-type cytochrome *c* in 0.1 M NaCl. (A) Difference between the titrations from pH 3 to 11 at 25 °C. (B) Difference between the titrations from pH 10 to 4.5 at 10 °C. The solid line is a least-squares theoretical fit assuming a single pK_a.

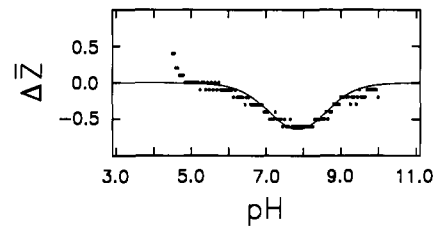

FIGURE 7: Difference titration curve of Ile-82 mutant minus wild-type cytochrome *c*. Proteins were titrated from pH 10 to 4.5 in 0.1 M NaCl at 25 °C. The solid line is the theoretical curve for a single ionizable group changing its pK_a from 8.5 to 7.2 upon mutation.

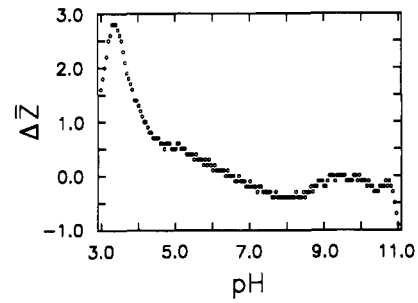

FIGURE 8: Difference titration curve of Ser-82 mutant minus wild-type cytochrome *c*. Proteins were titrated from pH 3 to 11 in 0.1 M NaCl at 25 °C.

Isoleucine-82 Mutant. Under the conditions that the potentiometric titrations were performed, solutions of the Ile-82 mutant develop turbidity below pH 4.5. Owing to this protein instability, only data from titrations between pH 4.5 and 10 can be analyzed. Examination of the difference between the titration curves of the Ile-82 mutant and the wild-type protein shows a trough centered at pH 7.9 (Figure 7). This result can be analyzed in terms of a change of pK_a in the wild-type protein from 8.5 to 7.2 in the Ile-82 mutant. At 10 °C, this trough is centered at pH 8.4 (data not shown) and can be analyzed in terms of an increase in 0.5 pK unit in both mutant and wild-type proteins upon decreasing the temperature from 25 to 10 °C.

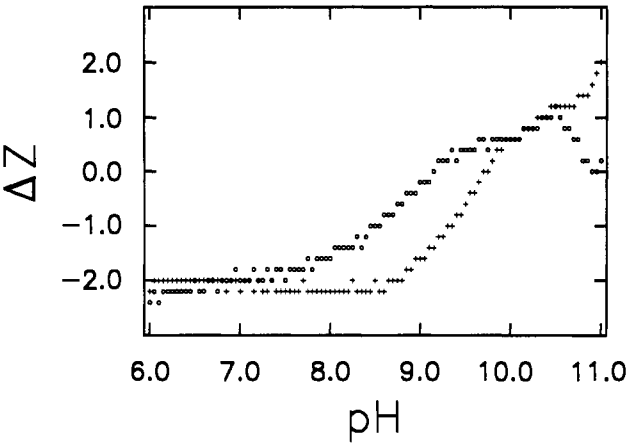

FIGURE 9: Difference titration curves of Phe-67 mutant minus wild-type cytochrome c at 25 °C (○) and 10 °C (+). Proteins were titrated from pH 6 to 11 in 0.1 M NaCl. The pH of zero net charge for this mutant was taken as 9.35 at 25 °C and 9.75 at 10 °C.

Serine-82 Mutant. The most prominent feature of the difference titration curve for the Ser-82 mutant minus the wild-type protein is the large deviation in the acid range (Figure 8). This observation is consistent with our finding that the acid pK for this mutant is 3.45 (n = 1.9) on the basis of spectrophotometric titrations at 525 nm. The Ser-82 mutant titrates a minimum of 2.5 groups more than the wild-type protein over the range pH 3–11 largely as the result of deviation at the acid end of the titration curve. In addition, the difference titration exhibits a minimum at pH 8.1 (Figure 8) that can be analyzed as a change in pK_a in the wild-type protein from 8.5 to 7.7 in the mutant (data not shown).

Phenylalanine-48 Mutant. In contrast, to the other mutant proteins studied here, no perturbations were observed in the titration curves of the Phe-48 relative to the wild-type protein at either 10 °C or 25 °C over the pH range 4.5–11. This finding suggests that in the wild-type protein Tyr-48 titrates outside of this range of pH and that substitution of phenylalanine for tyrosine at this position has no detectable effect on the pK_a's of other titratable groups within this pH range.

Phenylalanine-67 Mutant. The point of zero net charge of Phe-67 cytochrome c was found to be 9.35 (25 °C) and 9.75 (10 °C), or slightly lower than for the wild-type protein at either temperature. Difference titration curves (pH 6–11) calculated by subtracting the titration curve of the wild-type protein from that of the Phe-67 mutant for measurements at 25 and 10 °C are shown in Figure 9. Depending on the temperature and pH range considered, two to four more groups titrate in the wild-type protein than titrate in the mutant. The observed perturbation around pH 9 cannot be subjected to satisfactory numerical analysis in the absence of additional structural and functional information.

Discussion

The response of cytochrome c to changes in pH was first demonstrated by Theorell and Åkesson (1941) in their study of horse heart cytochrome c that reported the potentiometric titration curve and the pH dependence of the UV/visible spectrum of this protein. These investigators identified five pH-dependent forms of the protein that occur with pK_a values of 0.42, 2.5, 9.35, and 12.76. Since then, many potentiometric and spectrophotometric titrations of cytochrome c have been reported [e.g., see Bull and Breese (1966), Aviram and Schejter (1969), Shaw and Hartzell (1976), and Marini et al. (1980, 1981a)]. To our knowledge, however, yeast iso-1-cytochrome c has not been studied potentiometrically. With the recent determination of the three-dimensional structure of this protein to high resolution (Louie et al., 1988; Louie & Brayer, 1990) and the availability of a large number of point mutants constructed by site-directed mutagenesis (Pielak et al., 1985; Cutler et al., 1989; Rafferty et al., 1990) or available from application of classical mutagenic methods (Hampsey et al., 1986, 1988), characterization of the hydrogen ion titration curve of yeast iso-1-cytochrome c and selected mutants provides a means of evaluating the influence of specific amino acid residues on the electrostatic properties of this protein.

The titration curve for wild-type iso-1-ferricytochrome c establishes that 27–28 groups titrate between pH 3 and 11. On the basis of its amino acid sequence, iso-1-cytochrome c has a total of 42 titratable groups: 1 α-amino group, 1 α-carboxyl group, 2 heme propionate groups, 15 Lys, 3 Arg, 4 His, 8 Glu, 4 Asp, and 5 Tyr; a 16th Lys residue [Lys-72 (Delange et al., 1970)] is trimethylated. Although pK_a values have been determined for some of these residues in the yeast cytochrome (Cutler et al., 1989; Davies, 1989), insufficient experimental information is currently available to permit meaningful detailed discussion concerning the identities of those groups that do or do not titrate in the experiments reported here. Nevertheless, it seems reasonable to predict that the 14–15 ionizable residues that do not titrate in the pH range studied here include the 3 Arg residues, some subset of the Lys and Tyr residues, and the axial His ligand to the heme iron.

Hysteresis in hydrogen ion titrations of horse heart ferricytochrome c has been observed previously (Shaw & Hartzell, 1976; Marini et al., 1980). However, Marini and co-workers (Marini et al., 1980) report that exposure of the horse heart protein to acidic pH eliminates this behavior while we find that exposure of the yeast cytochrome to alkaline pH eliminates the hysteresis. Our finding that exposure of the protein to an alkaline pH no greater than pH 10 results in retention of hysteretic behavior suggests that the first alkaline conformational change (pK_a = 8.5) is not sufficient to change the electrostatic properties of the wild-type protein. Shaw and Hartzell (1976) interpreted their results to suggest that exposure of horse heart cytochrome c to alkaline pH results in a change in environment of a heme propionate group that produces a change in the pK_a of this group from 5.4 to 9.4. The difference titration that we calculate for yeast ferricytochrome c (Figure 1B) indicates that a well-defined pK shift of this type does not occur in yeast iso-1-ferricytochrome c.

While unambiguous, detailed interpretations of the titration curves obtained for the wild-type and mutant proteins are relatively difficult to develop, interpretation of difference titration curves calculated from them is more straightforward in many cases. For example, the pK_a shift from 6.5 to 6.9 determined from the difference titration curve shown for the Ala-38 mutant in Figure 5 correlates well with the shift in the pK_a of His-39 from 6.6 to 7.0 upon mutation of Arg-38 to alanine as measured by NMR spectroscopy (Cutler et al., 1989). The change in cytochrome c titration behavior on mutation of Arg-38 to His-38 is also readily understood. In this case, the titration curve of the mutant exhibits the presence of an additional group titrating between pH 3 and 11 as expected because arginine does not titrate in this pH range. Furthermore, the pK_a (8.2) of the new group present in the mutant protein (His-38) as determined from the difference titration curve is consistent with previous NMR studies that determined the pK_a of His-38 is greater than 7.5 and that the pK_a of His-39 in this mutant protein is the same as found in the wild-type cytochrome (Cutler et al., 1989). The acid region

of this difference curve is similar to that of the Ala-38 mutant and is, therefore, attributed to a difference in the pK of the acid isomerization between the His-38 mutant and wild-type protein.

Replacement of Phe-82 with Ile decreases the pH range over which the native conformation of the protein is stable to an even smaller range. This conclusion is based on our observation of precipitation of this mutant below pH 4.5 and our previous finding that the alkaline pK_a for this protein is 7.2. We interpret the difference titration curve shown in Figure 7B as arising from the change in alkaline pK_a from 8.5 in the wild-type protein to 7.2 in the mutant. On the other hand, replacement of Phe-82 with Ser produces greater alterations in the difference titration curve. In this case, the protein is not as extensively destabilized against acid denaturation, but the mutation nevertheless results in an elevation of the pK_a for the first acidic transition (to 3.45). Analogous to the result obtained for the Ile-82 mutant, the minimum observed at pH 8.1 in the difference titration of the Ser-82 mutant (Figure 8) is consistent with the reduction in pK_a for the alkaline transition from 8.5 in the wild-type protein to 7.7 for this variant (Pearce et al., 1989).

Two extremes in behavior are exhibited by substitutions of Phe for Tyr-48 or for Tyr-67. In the former case, the titration curve of the mutant protein between pH 3 and 11 is identical with that observed for the wild-type protein within experimental error. This finding indicates that the pK_a of Tyr-48 occurs with a value sufficiently greater than 11 that is not observed in the current experiments. On the other hand, replacement of Tyr-67 with Phe changes the titration curve of the yeast cytochrome c in a significant and complicated manner (Figure 9). Potential contributions to the complex effects of this mutation include elimination of Tyr-67 titration, perturbation of the alkaline pK_a in the mutant, and alterations in hydrogen-bonding interactions in the vicinity of the mutation that perturb the pK_a's of adjacent functional groups. Previous reports have suggested that the pK_a for Tyr-67 of horse heart cytochrome c is in the range of 10.0–10.5 (Czerlinski & Dar, 1971; Cronin et al., 1985), consistent with this assessment. Replacement of Tyr-67 with Phe in rat cytochrome c (Luntz et al., 1989) and in a semisynthetic horse heart (Wallace et al., 1989) cytochrome c possessing Phe-67 has been reported to increase the pK_a for the alkaline transition by at least 1 pK_a unit. A similar result has been obtained recently for the corresponding yeast mutant (G. Guillemette, unpublished results). Detailed evaluation of the possible effects of this mutation on the titration behavior of adjacent amino acid residues is deferred until analysis of the three-dimensional structure of the mutant cytochrome as determined by X-ray diffraction analysis is completed (A. Berghuis, G. Guillemette, and G. D. Brayer, unpublished results).

The thermodynamic properties of protein ionizable groups were first discussed in detail in Wyman's analysis of the titration curve of hemoglobin (Wyman, 1939). Since that time, a relatively small number of related thermodynamic studies have appeared for hemoglobin and other proteins [e.g., see Cohn and Edsall (1943), Antonini et al. (1963, 1965), Steinhardt and Reynolds (1964), Rossi-Bernardi and Roughton (1967), Perlman (1972), Parsons and Rafftery (1972b), Flogel and Biltonen (1975), Shiao and Sturtevant (1976), Marini et al. (1981b), and unpublished studies summarized in Matthew et al. (1985)]. The temperature dependence of the yeast iso-1-ferricytochrome c titration curve (Figure 3) in the alkaline pH range can be attributed in part to the increase in pK_a of histidine and lysine residues with decreasing temperatures. An additional contribution is made by the change in pK_a for the alkaline conformational change with temperature. The change in titration behavior produced by a change in temperature at low pH is attributed largely to the temperature dependence of the acidic conformational transition of cytochrome c based on the relative temperature independence of pK_a's of carboxylate groups (Cohn & Edsall, 1943).

The current results further demonstrate that the titration behavior of His-39 is highly temperature-dependent relative to model imidazole-containing compounds [e.g., see Agarwal and Perrin (1975) and Harris and Martell (1977)] and indicate an increase in the pK_a of 0.8 for this residue in both the wild-type and mutant proteins on reducing the temperature from 25 to 10 °C. This change in pK_a corresponds to an apparent heat of ionization of about 20 kcal/mol or around 3 times that normally observed for the ionization of an imidazolium group in proteins (Cohn & Edsall, 1943). A large apparent heat of ionization has also been observed for a histidine imidazolium group in ribonuclease A (Flogel & Biltonin, 1975). Interestingly, the pK_a of His-38 also increases significantly (+0.6 pK_a unit, ΔH_{app} = 15 kcal/mol) on decreasing the temperature from 25 to 10 °C as observed for His-39 in the wild-type and Ala-38 proteins. This is less surprising in that the arginine that His-38 replaces is almost completely buried and forms part of an extensive hydrogen-bonding network (Moore et al., 1986). A complete thermodynamic treatment of these and other mutants may highlight important ionizations in ferricytochrome c.

In addition to providing new information concerning the mutants of cytochrome c evaluated in this work, the present study illustrates the usefulness of classical proton titration curve analysis in the characterization of mutant proteins in general. Furthermore the instrumentation used in our work made it possible to use just one-tenth as much protein as used by most previous investigators and to collect a significantly larger number of data points during each titration. Through use of calculated mutation-induced difference titration curves as employed here, much of the usual difficulty in detailed interpretation can be greatly reduced. In such an analysis of mutants, information about either the substituted residue or the substituting residue can be obtained directly, as well as that concerning the remote electrostatic consequences of the mutation.

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Registry No. Arg, 74-79-3; Phe, 63-91-2; Tyr, 60-18-4; His, 71-00-1; Ala, 56-41-7; Ser, 56-45-1; Ile, 73-32-5; cytotchrome c, 9007-43-6.

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