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Journal of Chromatography A, 863 (1999) 137–146

JOURNAL OF CHROMATOGRAPHY A

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Cytochrome *c* unfolding on an anionic surface

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Received 2 June 1999; received in revised form 26 August 1999; accepted 9 September 1999

Abstract

It is now well accepted that the adsorption of proteins to solid supports sometimes involves surface-mediated unfolding. A detailed understanding of the adsorption and surface-mediated unfolding process is lacking. We selected a well studied protein, horse heart cytochrome *c*, and a weakly ionic support to examine some of the characteristics of protein adsorption under near-physiological conditions. We used high-performance liquid chromatography (HPLC) to investigate the effect of temperature on surface-mediated unfolding. Samples of cytochrome *c* were introduced to an anionic support, and a NaCl gradient was used to desorb the protein at different times and temperatures. The profiles and retention times were monitored to examine the adhesive properties of cytochrome *c* to the anionic support. We found that protein retention increased with time at temperatures as low as 0°C, and a significant loss of cytochrome *c* occurred between 55°C and 70°C. The loss of recovery of cytochrome *c* indicates irreversible surface-mediated unfolding. The changes in retention time may indicate more subtle transitions, including reversible surface-mediated unfolding of cytochrome *c* . These results suggest that perturbations in the structure as well as unfolding of cytochrome *c* can be detected at a lower temperature on an anionic surface than in solution thereby acting like a catalyst for protein unfolding.

Keywords: Ion-exchange chromatography; Cytochromes; Proteins

1. Introduction

Horse heart cytochrome *c* is composed of a single polypeptide chain covalently bound to a heme group. Cytochrome *c* is loosely bound to the outer surface of the inner mitochondrial membrane and functions as an electron carrier during oxidative phosphorylation by shuttling electrons from cytochrome reductase to cytochrome oxidase. Electrostatic interactions are essential for docking cytochrome *c* with its redox partners, which are embedded in the inner mitochondrial membrane. The surface of cytochrome *c* has a large cluster of positive charges due to lysine side chains, some of which correspond to negatively charged residues on cytochrome reductase and oxidase. The specific lysine residues (residues 8, 13, 25, 27, 72, 73, 79, 86 and 87) are found in a ring around the hydrophobic heme crevice and are highly conserved. Other highly conserved residues occupy positions associated with the heme group.

Cytochrome *c* is a relatively small protein, and in our experience, it is easily recovered from an anionic HPLC support. In a previous study, we observed a trend in which larger proteins unfolded more rapidly than small proteins [1]. We were curious whether cytochrome *c* would follow this trend by unfolding slowly and only under harsh conditions. We believe this trend is due to the flexibility of the protein and that smaller proteins, being generally more rigid than larger proteins at the same temperature, are less

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0021-9673/99/$ – see front matter
PII: S0021-9673(99)00975-9

likely to unfold on a surface. Certainly, this trend depends upon the functionality of the surface residues as well, but other factors being equal, we believed larger proteins would unfold more easily.

The chromatography of proteins has been intensely studied for the past several decades. However, chromatography research is most commonly focused on the separation capabilities of a media rather than examining their recovery. Ion exchange is believed to be a gentle process for separating proteins, particularly when compared to reversed-phase. In reversed-phase chromatography, proteins are often denatured as they elute from the system, whereas in ion-exchange, they are assumed to be in their native conformation [2–4]. However, we have observed loss of protein in ion-exchange, an indication that denaturation or unfolding has occurred [1,5]. Several researchers have shown that fibrinogen undergoes time-dependent conformational changes when bound to a polymeric surface [1,5–10]. We also examined the effects of temperature on the elution properties of cytochrome *c* in hydrophobic interaction chromatography [11,12].

Protein binding studies have shown that the structure and the associated properties of proteins are changed upon binding to a surface [13–18]. For example, protein adsorption causes loss of cooperative thermal denaturing [14]. In addition, protein adsorption often involves a slow unfolding process [13,16]. Sometimes different forms of a protein (holoprotein, apoprotein, etc.), which are structurally different in solution, appear similar upon binding [14]. It has been suggested that structural changes on negatively charged surfaces are the result of three events that occur upon binding [14]: (1) protonation of the surface residues, caused by a locally acidic environment induced by the negatively charged surface [19], (2) an increase in helical content, and (3) a loosening of the tertiary contacts. Event 1 may be important for proteins binding to negatively charged surfaces [14]; however, events 2 and 3 have been observed in proteins bound to hydrophobic material as well [17,18]. Furthermore, the changes observed in IgG bound to hydrophobic (non-polar) material were greater than those observed in silica (polar)-bound IgG [18].

Cytochrome *c* adsorption to a membrane-like array such as detergents or fatty acids induces a disruption of protein tertiary structure [20–23], which decreases thermal stability of the protein [20] and may increase activity [22]. Other more extensive alterations include the formation of a lipid-inserted denatured state that retains most of the secondary structure, but has lost most tertiary contacts, including Met80 heme ligation [24]. Pinheiro et al. suggested that the electrostatic interaction of cytochrome *c* with the membrane surface is responsible for the disruption of Met80 ligation and that the rate of this unfolding is limited by disruption of the hydrophobic core [24]. The secondary structure of bound cytochrome *c*, however, is almost identical to the solution structure [22,23]. Secondary structural changes that occur include a slight reduction in helical content of membrane bound cytochrome *c* [21], although an increase in alpha helical content is observed for apocytochrome *c* upon binding to phospholipid [25].

Temperature studies on bound cytochrome *c* have shown a decrease in the temperature required for denaturation from ~82°C to ~55°C [21,23]. Additional temperature-induced transitions have been observed between 22°C and 29°C for membrane-bound cytochrome *c* [21] and between 50°C and 60°C for cytochrome *c* in solution [23,26]. The structural transitions that occur at 'low temperature' on a membrane may have been specific for the lipids used in the membrane for that study. The transition that occurs at the lower temperature in solution is associated with an opening of the heme crevice, similar to the structural transition seen upon adsorption. The bound deformed protein was more 'disordered' than denatured cytochrome *c* in solution [21]. A gradual loosening of tertiary contacts between the temperatures of 10°C and 40°C of bound cytochrome *c* was observed in the bound position but not in solution [21].

2. Experimental

2.1. Materials

The HPLC (Hewlett–Packard, Palo Alto, CA, USA, Model 1090) was equipped with a diode-array detector, column heater, and autosampler. The pascal-based Hewlett–Packard Chemstation was used for recording chromatograms and integrating peaks.

The anion-exchange column used was a sulfopropyl nonporous TSK Gel column (particle size=2.5 μm) (TosoHaas Montgomeryville, PA, USA). All water was deionized and purified using a Milli-Q water system (Milliport, Bedford, MA, USA). Buffer A contained 5 m*M* Trizma base (reagent grade, Sigma, St. Louis, MO, USA) brought to pH 7.4 using concentrated HCl. The solution was then filtered through a 0.45-μm membrane (Millipore) and degassed with ultra high purity helium (Oxarc, Spokane, WA, USA). Buffer B was identical to Buffer A, except that it also contained 1.0 *M* NaCl (≥99% purity, ACS grade, Sigma). Cytochrome *c* (>95% purity, Sigma) was mixed fresh daily in a solution of 1 mg/ml protein in Buffer A.

2.2. Methods

All HPLC gradients (with one exception) began with Buffer A and ended with Buffer B. The anion-exchange column and the cytochrome *c* sample were placed in an ice bath for 0°C sorption tests. The column heater chamber was used on the HPLC system, and the sample was placed in the chamber with the column for higher temperature experiments. The flow-rate was kept at 0.5 ml/min for all experiments. The injection volume for each experiment was 25 μl of sample.

The elution parameters varied slightly from one gradient to the next. The variable t_1 (time between injecting the sample and the beginning of the linear gradient) was the only variable between gradients 2 through 7. Table 1 shows the values of t_1 for each gradient used in this study. 'Gradient 1' was an isocratic condition using Buffer B (1.0 *M* NaCl). With 1.0 *M* NaCl, cytochrome *c* should not bind at all to the anionic support. All gradients (except gradient 1) consisted of the buffer increasing linearly in NaCl concentration from 0 *M* to 1 *M* over 5 min (rate=0.2 *M*/min). After each gradient was completed, the column was washed for 5 min with Buffer B to ensure elution of all recoverable cytochrome *c* and accurate peak integration. Each chromatogram was repeated four times. Although our gradients were run from 0 to 100% B, the samples eluted near the middle of the gradient, so small perturbations near the ends of the gradient were unimportant for this work. We typically run gradients in this manner but are careful to construct them so that the analyte eludes in the middle of the gradient. Part of the reason for this has to do with minimizing waste.

Table 1
Values of t_1 for each gradient used[a]

Gradient No.	t_1	Residence (min)
1	N/A	~0
2	0	2.5–3
3	2	4.5–6
4	5	7.5–9.5
5	7	9.5–11.5
6	10	12.5–15
7	12	14.5–17

[a] Gradients used in this study are indicated by numbers 1–7. The values of the variable t_1 refer to the delay between sample injection and initiation of the linear gradient. For each gradient, the range of residence times was that observed for oxidized and reduced cytochrome *c*. No gradient was used for gradient 1. The sample passed isocratically and unretained through the ion-exchange column in the presence of 1.0 *M* NaCl.

Absorbance at 280 nm and 408 nm was recorded with a reference wavelength of 550 nm. The absorbance at 408 nm was used to compare elution of cytochrome *c* from the column under varying experimental conditions. The absorbance at 280 nm was used to verify the 408-nm data. Absorbance was recorded to monitor loss of the protein and to ensure that protein and the porphyrin ring were both eluting from the column. Unfolded protein was defined as that which was bound to the column such that it did not elute under these gradient conditions.

Peaks were integrated using the HP Chemstation. The area of the cytochrome *c* peak at 408 nm after passing through a low dead volume connector corresponded to 100% recovery. The area of the cytochrome *c* peak at 408 nm after passing through the column corresponded to the recovery of the protein under each condition. Therefore, recovery of cytochrome *c* was calculated as the ratio of $(\text{area}_{\text{gradient No.}})/(\text{area}_{\text{dead volume}})$. The data were statistically analyzed using two-factor analysis of variance.

Retention time t_R was calculated as the time between the injection of the protein into the system and the elution of the protein. Residence time is the time the protein spent on the column and was calculated by subtracting the dead volume t_R from

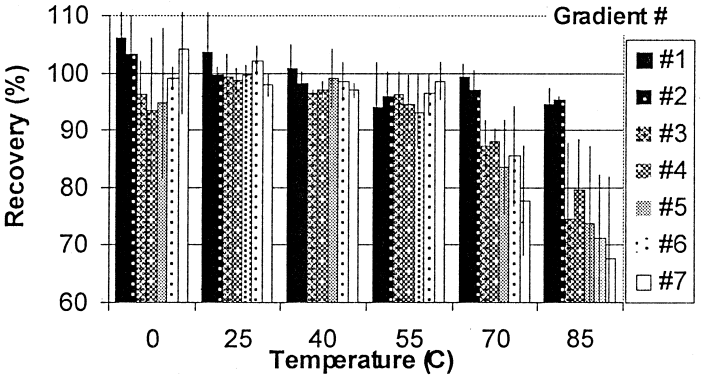

Fig. 1. Recovery of cytochrome *c* as a function of temperature and residence time. Error bars indicate standard deviation for four analyses.

the observed t_R. The prefix 'adjusted' added to the beginning of t_R or 'residence time' signifies that t_1 (Table 1) was subtracted from either the residence time or t_R. These adjusted values show relative binding strengths and correspond roughly to the ionic strength of the mobile phase.

3. Results and discussion

3.1. Recovery

The irreversible unfolding of cytochrome *c* appeared to be slow on the sulfopropyl (anionic) surface. Gradients 1and 2 resulted in almost no loss of protein over the 0 to 85°C temperature range. Gradient 1 was expected to show no unfolding because of the exclusive use of Buffer B (1.0 *M* NaCl). Gradient 2, however, also showed essentially no loss of protein over the 0 to 85°C temperature range. Fig. 1 shows that there was a consistent loss in recovery when using gradients 3 through 7 that occurred at the temperatures of 70°C and 85°C. At these two temperatures, there was no statistical difference in the recovery of cytochrome *c* between gradients 1 and 2 or in the recovery of cytochrome *c* between gradients 3–7 (although the recovery did continue to decrease slightly as residence time increased). Therefore, in Fig. 2, the data are grouped according to gradient used. This figure shows the decrease in recovery observed at 70°C and 85°C when the cytochrome *c* was bound to the column for at least 5.0 min. Table 1 illustrates that the use of gradient 2 resulted in residence times of 2.5 to 3.0

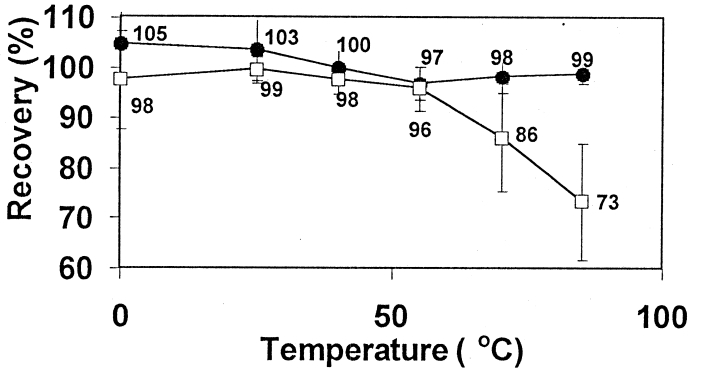

Fig. 2. Recovery of cytochrome *c* as a function of temperature and residence time. Error bars indicate standard deviation for four analyses. Shown are recoveries from very short (≤ 4.5 min) residence time ●, and longer residence times □. The two-factor analysis of variance (ANOVA) indicated that statistically, the data could be grouped in this manner (Table 1).

min, while gradient 3 resulted in residence times of 4.5 to 6.0 min. Furthermore, at these temperatures, gradient 3 resulted in residence times of 5.0 to 6.0 min. Therefore, a slow process that took longer than 3 min, but less than 5 min, seems to have been responsible for the observed loss of protein at higher temperatures (ca. 60°C to 85°C).

As a control, we used a long piece of tubing and low flow-rate to hold cytochrome *c* in the column heater while it was kept at various temperatures and periods of time. These control data always gave high (95–105%) recoveries, even when 85°C and 8 min residence time were used. This suggests the losses observed were not caused by temperature alone, that adsorption to the sorbent was required.

3.2. Chromatography

Fig. 3 shows elution profiles of cytochrome *c* when gradients 2 and 7 were used. These profiles correspond to the shortest and longest residence times that cytochrome *c* spent on the anionic surface. The first peak to elute from the column had an absorption spectrum identical to that of reduced cytochrome *c* (characterized by a maximum peak near 410 nm, a broad peak ~315 nm, a peak near 520 nm and 550 nm) [22]. The second peak to elute had an absorption spectrum identical to oxidized cytochrome *c* (a maximum near 410 nm with a shoulder near 360 nm and a broad peak near 530 nm) [22]. The chromatogram displayed the reduced cytochrome *c* as a shoulder of the oxidized cytochrome *c* peak at low temperatures. As the temperature increased, the resolution between these peaks increased. At the highest temperatures tested, the reduced cytochrome *c* peak was absent. The cytochrome *c* originated in the oxidized form. Therefore, cytochrome *c* reduction took place during the chromatographic separation. One possible explanation for this conversion is depletion of oxygen in the mobile

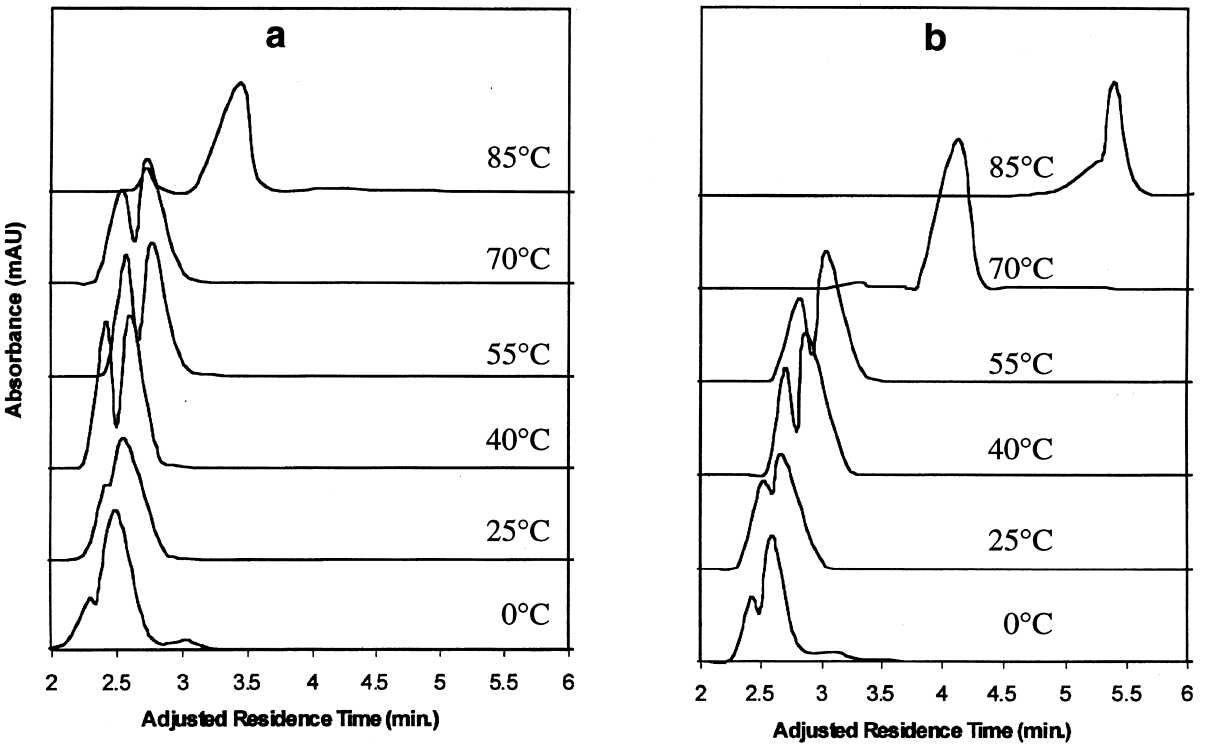

Fig. 3. Representative chromatographic profiles of cytochrome *c* from the cation-exchange column using gradients 2 (a) and 7 (b) at various temperatures. Conditions were those described in Table 1. Adjusted residence time subtracted t_1 (Fig. 1) from the elution time to normalize the data to the retention time relative to the start of the linear gradient.

phase as we spurged with helium. Another possible explanation is conversion through protein–column interactions. We did not attempt to examine this conversion process further in the present study.

As described in the Methods section, the adjusted retention time t_R reflects the salt concentration required to elute cytochrome *c*. A greater adjusted t_R showed that more NaCl was required to force the protein off the anionic surface. A small, but repeatable, continual increase in adjusted t_R using gradient 2 was observed over the temperatures between 0° and 70°C, but at 85°C, the adjusted t_R showed more variability (Fig. 3a). Using gradient 7, similar trends were observed between 0°C and 55°C; however, a greater increase in adjusted t_R was seen between 55°C and 70°C as well as between 70°C and 85°C (Fig. 3b).

Fig. 4 shows the chromatographic behavior of cytochrome *c* at 0°C, 40°C, and 85°C on an anionic surface for different residence times. At 0°C, there was only a slight increase in the adjusted t_R of cytochrome *c* over the residence times tested. At 40°C, there was a more noticeable increase in adjusted t_R as cytochrome *c* was held on the surface for longer periods of time. Furthermore, the first peak constituted a larger portion of the total peak area at 40° than at 0°. At 85°C, there were even greater increases in adjusted t_R as cytochrome *c* was held on the anionic surface for longer periods of time. We generally observed a continual increase in adjusted t_R and decrease in recovery at higher temperatures.

3.3. Retention time

Fig. 5 shows how the adjusted t_R of reduced cytochrome *c* changed over the range of experimental conditions. Not all chromatograms showed two separate peaks. At low residence times and low temperatures, the first peak was observed as a shoulder; however, assignment of a retention time to the shoulder was not attempted. At high temperatures (70°C to 85°C) and long adjusted t_R values (>3.5 min), the first peak disappeared completely.

Fig. 6 shows the changes in the adjusted t_R of oxidized cytochrome *c* over the range of experimental conditions. Two-factor analysis of variance (95% confidence) was performed on adjusted t_R values to

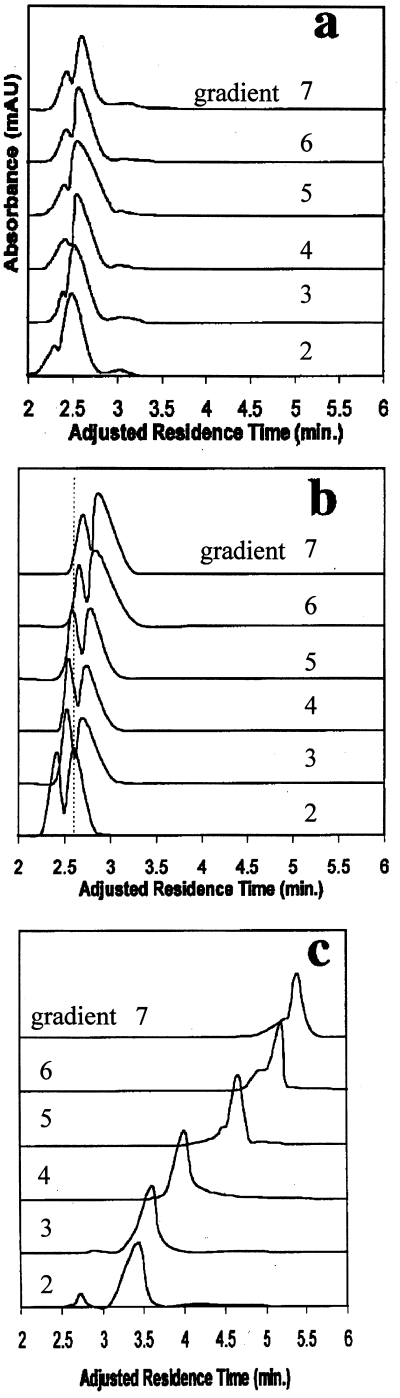

Fig. 4. Representative electron profiles of cytochrome *c* from the cation-exchange column 0°C (a), 40°C (b), and 85°C (c) and gradient conditions as defined in Table 1. Adjusted residence times were as described in Fig. 4.

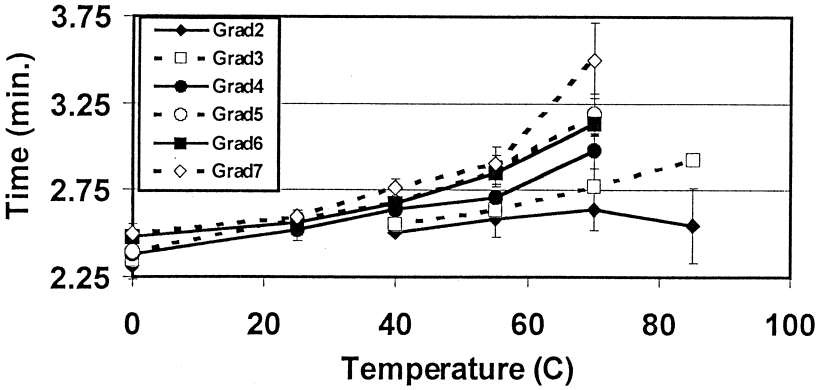

Fig. 5. The effects of temperature and t_1 on the adjusted residence time of reduced cytochrome *c*. Adjustment retention times are the values corresponding to the ionic strength of the mobile phase required to elute the cytochrome *c* from the anionic support. They have been derived by subtracting t_0 (dead volume) and t_1 (Table 1) from the retention time. Not all chromatograms had a separate reduced peak (either from being a shoulder of the oxidized peak at lower temperatures or from disappearing at higher temperatures). Error bars indicate standard deviation.

determine the statistical significance of observed differences. The adjusted t_R values of the chromatograms obtained were each significantly different, with 95% confidence, from one another. The adjusted t_R vales increased with increasing temperature and with hold times. The adjusted t_R increased gradually from 25°C to 55°C for all the gradients used. The adjusted t_R values increased sharply between 55°C and 70°C and continued to increase similarly between 70°C and 85°C. The adjusted t_R when gradient 3 was used showed less of an increase between 55°C and 70°C, but a comparably rapid increase was observed between 70°C and 85°C. The adjusted t_R of gradient 2 showed no statistically significant increase; however, the possibility of an increase at 85°C cannot be discounted completely, as the data at this temperature were highly variable.

Fig. 7 shows the relative increases between the adjusted t_R values of oxidized and reduced cytochrome *c* . It is interesting to note that between 25°C and 40°C, adjusted t_R showed increases for each individual peak; however, the differences between the two peaks were identical. At 55°C, the two peaks began to separate, and at 70°C, the differences in retention were noticeable, but more variable. At 85°C, there was very little data for the first peak, and

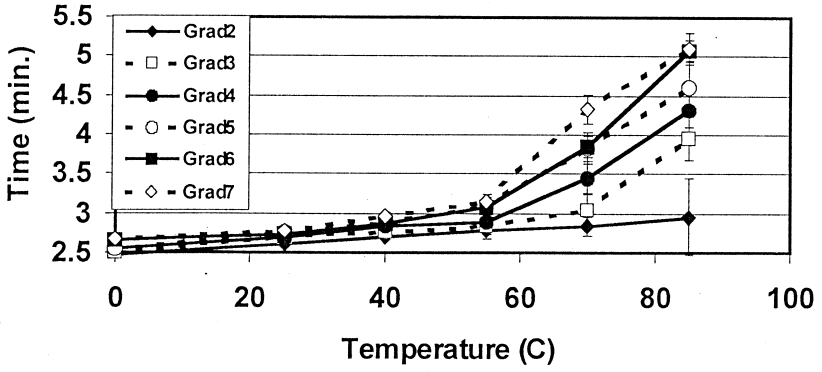

Fig. 6. The effects of temperature and t_1 on the retention time of oxidized cytochrome *c*. Adjusted residence times are the values corresponding to the ionic strength of the mobile phase required to elute the cytochrome *c* from the anionic support. They have been derived by subtracting t_0 (dead volume) and t_1 (Table 1) from the retention time. Error bars indicate standard deviation.

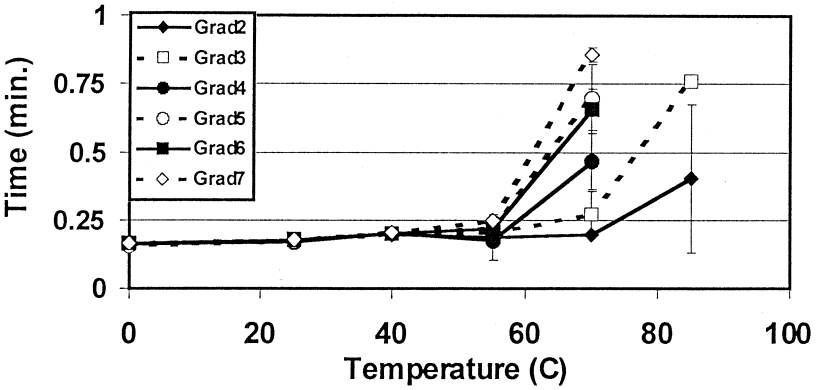

Fig. 7. The effects of temperature and t_1 on the difference of retention times of reduced and oxidized cytochrome *c*. The time between elution of oxidized and reduced cytochrome *c* changes with temperature. The values for gradients 2–7 are shown. Not all chromatograms had a separate reduced peak (either from being a shoulder of the oxidized peak at lower temperatures or from disappearing at higher temperatures). Error bars indicate standard deviation.

therefore only the shortest residence times can be shown on Fig. 7. However, at this temperature, it was clear that the two peaks continued to separate.

Considerable uncertainty still exists about the general principles governing protein adsorption [27]. Many very different equilibrium states can exist under adsorption conditions, which frequently gives rise to competition between reversible and irreversible binding reactions. A model based on this type of competition was recently applied to albumin adsorbed on $Si(Ti)O_2$ surfaces [28]. A similar point of view provides a qualitative interpretation of our results.

We propose a model that involves three types of adsorbed species, cytochrome *c* in its native conformation, a reversibly unfolded state, and a denatured irreversibly unfolded state. The reversibly unfolded state on the surface might be similar to the solution phase molten globule (MG) state in the presence of GdmHCl [29] or the 'A-state' at low pH and high ionic strength [30]. This state may also be associated with a possible unfolding intermediate observed for cytochrome *c* in hydrophobic-interaction chromatography [31] within the temperature range of 15°C to 40°C.

The increases in retention time found in our study between 25°C and 40°C may correlate with changes in retention observed in hydrophobic interaction chromatography between 25°C and 40°C [32]. Greater retention time is associated with a larger number of contacts between the protein and the chromatographic surfaces. A gradual loosening of tertiary structure has been observed for bound cytochrome *c* [21] between the temperatures of 10°C and 40°C. It is reasonable to expect this reversible unfolding to increase the number of contacts points and the retention time, as observed in our experiments.

Recently, Wang et al. [33] reported progressive unfolding of cytochrome *c* in solution observed by limited proteolysis. This method can identify the secondary structural elements that are unfolding at a given temperature. Changes in 280- and 420-nm optical adsorption that begin at 40°C are correlated with a cut in a loop structure (residues 36–61) at the base of the heme cavity. As temperature increases, other loops in the native cytochrome *c* structure become susceptible to proteolytic digestion. CD profiles and proteolytic digestion indicate the melting of helical regions begins at temperatures in excess of 80°C. We propose that a similar multi-state thermal unfolding occurs in our experiments and gives rise to incremental increases in retention time as the temperature increases. Therefore, these conformational changes must occur at a lower temperature, when cytochrome *c* is in contact with a surface. The fact that bound cytochrome *c* denatures at 55°C [21,23] compared to a value of 83°C in solution [26,33] supports this hypothesis.

We suspect that eluted cytochrome *c* was in a near-native state. The protein that remained bound to

the column was probably more fully denatured. This interpretation is consistent with the disappearance of the first peaks at higher temperatures (Figs. 3 and 4). The gradual increase in retention time observed with increasing temperature and hold time is also consistent with the stronger binding of a more fully denatured protein. As unfolding increases, the protein expands, giving it an opportunity to bind at more sites on the rigid surface of the column packing material and therefore, more strongly.

It was a challenge to force cytochrome *c* to unfold on our ion-exchange support. Under most chromatographic conditions, cytochrome *c* eluted fully with 100% recovery (Figs. 2 and 3). Only when the temperature was raised and the residence time increased would some portion of the protein remain bound to the sorbent. Even under the most extreme conditions, we were able to observe only a small percentage of the cytochrome *c* unfolded on the sorbent (30% maximum). It is not fully clear why this relatively small percentage of bound protein unfolded, however, it may be related to the biological function of cytochrome *c* near a lipid membrane. That is, cytochrome *c* may function under conditions that would be denaturing to many other proteins.

The resistance to surface-mediated unfolding exhibited by cytochrome *c* is due, in part, to its small size [1]. Large multiple-domain proteins, like fibrinogen, can interact with a surface at multiple binding sites simultaneously without major deviations from their native conformation. In its native conformation, cytochrome *c* may be limited to one or only a few contact points. Reversible unfolding increases the number of contact points and favors the development of the irreversible surface-bound state.

Fig. 5 shows that, given ample time, fluctuations in protein conformation allow cytochrome *c* to bind more strongly. The small increase in retention time in response to a longer residence time on the surface shows that stronger binding can develop over time even at 0°C. Increases in retention with longer time on the column became more drastic as the temperature was raised. If similar retention times correspond to similar structural changes, then either residence time or temperature can produce these structural modifications and require a similar salt concentration to desorb the protein. Examples are gradient 3 at 70°C compared to gradients 6 and 7 at 40°C as well as gradient 3 at 85°C compared to gradients 5 and 6 at 70°C in Fig. 6.

The heme group is a major source of conformational stability for cytochrome *c* in solution [34]. This stabilizing effect, which is present in both the native and MG states, is derived from two types of interactions, the axial iron ligands and the hydrophobic contribution of the porphyrin ring. These interactions undoubtedly play a role in cytochrome c's resistance to surface-induced unfolding. In the native structure, the MG state, and some early folding intermediates, N- and C-terminal helices together with the heme form a compact hydrophobic core. In solution, rearrangements within this core (Met80 replaced by a non-native histidine) occur at a much lower temperature (50–60°C) than the loss of secondary structure (about 80°C) [26,33]. The former is reversible but is kinetically unfavorable. Dissociation of the non-native His ligand under refolding conditions is much slower than the corresponding rates of non-native His ligand association under denaturing conditions [35]. This implies that protein refolding, and not solely unfolding, probably has a significant impact on the recovery of cytochrome *c*.

Our findings suggest that conformational changes similar to those observed in solution occur at a lower temperature when cytochrome *c* is adsorbed. This result can be viewed as the surface acting similar to a catalyst for unfolding. The difference is that a catalyst only reduces the activation energy. A surface can lower the energy state of the product (unfolded protein) relative to the energy of unfolded conformations in solution. Lowering product energies can have a secondary effect of lowering the transition-state and the activation energy. Another way that a surface might enhance the formation of unfolded states would be to serve as a sink for strain energy. Conformational changes in solution resulting from random thermal fluctuations usually have large strain energies that favor transitions back to the native state. A pathway to rapidly dissipate this energy (i.e., a surface) would help to overcome kinetic barriers to unfolding. Even though surface-mediated unfolding is a multi-step process, these analogies with rate theory for simple reactions may provide insights for relating the behavior of adsorbed proteins to their solution properties.

Acknowledgements

Pacific Northwest National Laboratory is operated for the US Department of Energy by Battelle under Contract DE-AC06-76RLO 1830. This work was supported by the US Department of Energy, Laboratory Directed Research and Development Medical Technologies and Systems Initiative. The authors thank Dave Springer, Keith Dunker, Joe Marx, for useful discussions. Also, special thanks are given to Debbie Sklarew, who performed some of the early experiments that led to this study and Adrienne Williams who reproduced our results with repurified protein.

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