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Ribose 5-Phosphate Glycation Reduces Cytochrome *c* Respiratory Activity and Membrane Affinity

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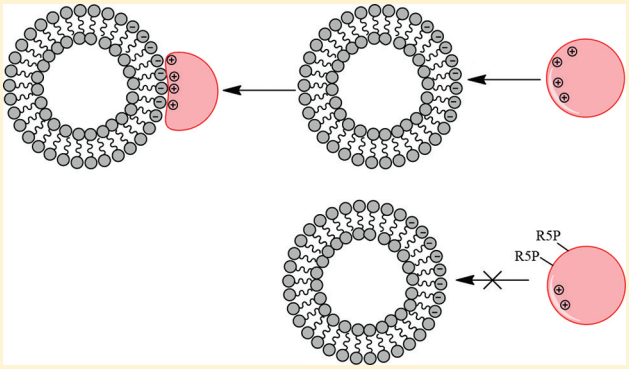
Supporting Information

ABSTRACT: Spontaneous glycation of bovine heart cytochrome *c* (cyt *c*) by the sugar ribose 5-phosphate (R5P) weakens the ability of the heme protein to transfer electrons in the respiratory pathway and to bind to membranes. Trypsin fragmentation studies suggest the preferential sites of glycation include Lys72 and Lys87/88 of a cationic patch involved in the association of the protein with its respiratory chain partners and with cardiolipin-containing membranes. Reaction of bovine cyt *c* with R5P (50 mM) for 8 h modified the protein in a manner that weakened its ability to transfer electrons to cytochrome oxidase by 60%. An 18 h treatment with R5P decreased bovine cyt *c*'s binding affinity with cardiolipin-containing liposomes by an estimated 8-fold. A similar weaker binding of glycated cyt *c* was observed with mitoplasts. The reversal of the effects of R5P on membrane binding by ATP further supports an A-site modification. A significant decrease in the rate of spin state change for ferro-cyt *c*, thought to be due to cardiolipin insertion disrupting the coordination of Met to heme, was found for the R5P-treated cyt *c*. This change occurred to a greater extent than what can be explained by the permanent attachment of the protein to the liposome. Turbidity changes resulting from the multilamellar liposome fusion that is readily promoted by cyt *c* binding were not seen for the R5P-glycated cyt *c* samples. Collectively, these results demonstrate the negative impact that R5P glycation can have on critical electron transfer and membrane association functions of cyt *c*.



As the only nonintegral protein in the electron transport chain, cytochrome *c* (cyt *c*) associates with its respiratory partner cytochrome oxidase (cyt aa_3) by use of a group of lysines rimming the heme edge. The association of this positive region with corresponding negative groups contained on the surface of cytochrome oxidase allows the heme-to-heme contact needed for efficient electron transfer.[1−3] Important lysines, such as Lys87, that function in this patch can be shifted away from the region upon the binding of ATP at a neighboring cationic patch (positioned near Arg91).[4] Consequently, ATP is proposed to serve as an important inhibitory regulator of respiratory activity in the cell.[5−7]

Cyt *c* also has an attraction to the outer leaflet of the inner mitochondrial membrane.[8−10] This membrane contains high levels of cardiolipin, a negatively charged lipid component that associates with the highly positive cyt *c*.[10,11] Previous research has identified three different regions on the cyt *c* surface that may be involved in membrane binding: the A-site centered around Lys72/73[12] that may also contain Lys86, Lys87, and Arg91;[13] the C-site situated in the surface region at Asn52;[12,14] and the L-site located in the vicinity of Lys22 and Lys27.[12] While there is still some confusion about which site(s) is involved in cardiolipin association, the binding event appears to involve the insertion of at least one[13] and more likely two acyl chains[12,15] of cardiolipin into the interior of the cyt *c* molecule. It has been postulated that one of these acyl chain insertions causes a shift of the Met80 from its coordination with the iron of the heme group as indicated by the transition of reduced cyt *c* from a low-spin form to a high-spin form.[13,15] At some point in this process, the peroxidase activity of cyt *c* is activated, which oxidizes membrane lipids triggering events that lead to the exit of cyt *c* from the intermembrane space and its subsequent initiation of apoptosis in the cytosol.

Spontaneous glycation of protein amines (N-termini, Lys, and Arg) by sugars is a reaction that can lead to protein modification and dysfunction. Using the sugar glucose, the process is typically slow, requiring weeks and months to generate significant protein modification. In the case of long-lived proteins like hemoglobin, this creates modified protein versions (e.g., HbA_{1c}) with a partial or complete lack of function. The reaction rate is faster for sugars with higher proportions of the acyclic form and for sugars containing catalytic groups. Because of its relatively favored

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acyclic form and its correspondingly high glycation rate, we have investigated the reaction of the pentose phosphate pathway intermediate ribose 5-phosphate (R5P) with free and protein amine groups. R5P has glycation rates ~100 times greater than that of relatively nonreactive sugars such as glucose.[16,17] Containing a negatively charged phosphate group, R5P may have particularly high reactivity toward highly cationic proteins such as cyt *c* (pI ~ 10). Recently, we demonstrated the high potential of R5P for reduction of cyt *c*.[18] A portion of the reducing power comes from the synthesis of superoxide (O_2^-) during a glyoxidation reaction generated at the cyt *c* surface, while some of the cyt *c* reduction appears to be due to a dicarbonyl product of R5P. This process in vivo is greatly important as an increased level of activation of the pentose phosphate pathway in cancer cells (as part of the Warburg effect) is somehow responsible for keeping cytosolic cyt *c* in a reduced form, thus making it apoptotically inactive.[19] Glutathione appears to be involved in the reduction process, but R5P, shown to have a concentration increased by 2−10-fold in breast tumor cell lines,[20] could also play a reductive role. We are currently investigating the impact that higher levels of R5P and other pentose phosphate intermediates have on the initiation of the apoptosis process.

In this study, we report on the effect of R5P glycation on cyt *c*'s electron transfer activity and its membrane binding properties. Four lysines on the protein appear to be preferred sites of modification. Alteration of these lysines results in cyt *c* being less able to transfer electrons to cytochrome oxidase. In addition, the affinity of R5P-glycated cyt *c* for cardiolipin-containing liposomes and prepared mitoplasts is significantly decreased. These results suggest that glycation events can alter critical functional groups on cyt *c*, leading to impaired functions in respiration and apoptosis.

MATERIALS AND METHODS

Materials. Bovine heart cyt *c*, yeast cyt *c*, egg yolk phosphatidylcholine, bovine heart cardiolipin, R5P, adenosine triphosphate (ATP), glucose, and proteomics grade trypsin were purchased from Sigma-Aldrich Chemical Co. and used without further purification. All other reagents were of the highest purity available. The sequence numbering used for bovine and yeast cyt *c* is based on the numbering used for horse heart cyt *c*.[21]

Glycation Reactions. Typically, solutions containing cyt *c* (1.0 mg/mL, 82 μM) and R5P (10 mM) were combined, adjusted for pH (pH 7.4) if necessary, and incubated at 37 °C for periods ranging from 2.5 h to 6 days. For the glucose reactions, the incubation mixtures contained 125 mM glucose and 10 mM phosphate. In some cases, incubation samples were frozen (−20 °C) for short periods prior to analysis.

Cytochrome Oxidase Activity. Soluble cytochrome oxidase (COX) was prepared from Keilin-Hartree particles as described by Kuboyama et al.[22,23] The final soluble COX preparation was frozen at −20 °C in aliquots less than 2 weeks prior to use. The cytochrome oxidase activity was determined by monitoring the rate of oxidation of reduced cyt *c* by the soluble COX preparation at 550 nm. Briefly, ferri-cyt *c* (1 mg/mL; untreated or glycated with R5P) was reduced by 0.5 mM dithiothreitol for 15 min. Cyt *c* samples (150 μL) were then combined with 40 mM phosphate buffer (pH 7.0, 50 μL) in a microwell. Small amounts (1−5 μL depending on activity) of the soluble COX preparation were added to the well, and the absorbance at 550 nm was monitored over 2 min at room temperature. One unit of COX activity is defined as the ability of the enzyme to convert 1 μmol of reduced cyt *c* ($\Delta\varepsilon_{550}$ = 19.6 mM^{-1} cm^{-1}) to the oxidized form per minute of reaction. Results were analyzed with a Student's *t* test.

Cyt *c* Glycation Analysis by Liquid Chromatography and Mass Spectrometry (LC−MS). The attachment of R5P molecules to bovine heart cyt *c* can be monitored with reaction time by LC−MS. Incubation samples (37 °C, 0−24 h) were injected into an Agilent Series 1100 MS/MSD Trap XCT Plus LC-MS instrument, and elution on a Zorbac C18 column was conducted using a 1:1 water/acetonitrile mixture [both with 0.1% trifluoroacetic acid (TFA)]. The compounds eluting via LC were analyzed by ion trap MS using a target *m*/*z* of 1500. The molecular masses of the cyt *c* products were determined by charge state analysis.

Cyt *c* Trypsin Digestion. LC−MS analysis of tryptic digests was conducted with untreated cyt *c* and cyt *c* that had reacted with R5P (10 mM) at 37 °C for up to 6 days. Cyt *c* solutions denatured in 6 M urea for 1 h at 50 °C were microfiltered through Amicon Microcon 10000 molecular weight cutoff (MWCO) filters to remove urea and R5P. The retentate was retained in 0.5 M ammonium bicarbonate buffer, and proteomics grade trypsin was added at a 1:25 protein ratio and the mixture allowed to incubate for 16−20 h at 37 °C. All samples were analyzed using the Agilent ion trap LC−MS instrument with a Zorbax C18 reverse phase column. Gradient elution (0.5 mL/min) of the digested pieces was accomplished starting at 100% water and increasing linearly to 70% acetonitrile over a 50 min period. (Both solutions contained 0.1% TFA.) The target mass was set at an *m*/*z* of 900. Extracted ion chromatograms (EICs) of the eluting peptides were generated and integrated. This method allowed full coverage of the protein, although certain potential multiple-cleavage products where sequential lysines or a lysine-arginine pair was present (i.e., Lys6-Lys7, Arg38-Lys39, Lys72-Lys73, Lys86-Lys-87-Lys88, and Lys99-Lys100) tended to show trypsin cleavage preferentially at one site or the other. The intensity of each EIC peak of cyt *c* treated for various lengths of time with R5P was compared to the intensity of the corresponding peak with no R5P incubation to evaluate the decreased level of trypsin action at the site due to glycation modification. Criteria were established for designating a particular lysine or arginine as a highly probable target of R5P glycation (see the Supporting Information). It is important to note that the N-terminus of cyt *c* is acetylated and therefore not a glycation target.

Liposome Preparation. Phosphatidylcholine (egg yolk) and cardiolipin (bovine heart) were each prepared at a concentration of 2.0 mg/mL in chloroform. The appropriate proportions were dispensed into a brown vial, and a stream of N_2 gas was directed at the surface until the chloroform had fully evaporated (~1 h). An appropriate amount of 10 mM HEPES buffer (pH 7.4) was added, and the vial was vortexed until the lipid on the walls of the vial had disappeared (~2 min). Unless noted, a 1680 μM total lipid solution was prepared with a cardiolipin concentration of 20%. These large multilamellar vesicles (estimated in size to be 0.5−15 μm) were used for some binding experiments (termed "multilamellar"). Most of the spectral change experiments were performed using a unilamellar liposome preparation.[13] The multilamellar solution was subjected to five rapid freeze−thaw cycles followed by vortexing for 1 min. This solution was then filtered 13 times through a 0.1 μm polycarbonate membrane at 37 °C using a mini-extruder (Avanti Polar Lipids, Inc.). This treatment gives unilamellar liposomes (termed "unilamellar") that were ~100 μm in diameter. Unilamellar liposomes were stored at 4 °C and used

within a few days of preparation. All molarity ratios are reported as cyt *c* to total lipid concentration.

Preparation of Beef Heart Mitoplasts. We prepared mitoplasts were prepared[6] by homogenizing bovine heart tissue with a Potter-Elvehjem at 4 °C in a 10-fold volume of buffer [250 mM sucrose, 10 mM Tris, and 2 mM EDTA (pH 7.2)], centrifuging the mixture at 3000*g* to pellet the nuclei and membranes, and centrifuging the subsequent supernatant at 12000*g* to pellet the mitochondria. The outer membrane was ruptured, and the loosely bound cyt *c* was dislodged by subsequent treatments with 15 and 150 mM KCl (with 12000*g* centrifugations). The final mitoplast preparation (in 10 mM KCl) was stored at 4 °C until it was used.

Assessment of Glycation at the ATP Binding Site. The interaction of R5P with residues at the ATP binding site was investigated by two methods. First, a sample of cyt *c* (1.0 mg/mL) and R5P (10 mM) with and without ATP (10 mM) were incubated at pH 7.4 and room temperature for periods of up to 5 h. At various time intervals, samples were removed and subjected to LC−MS analysis using an Agilent LC−MS instrument. LC separation employed an isocratic 1:1 water/acetonitrile (with 0.1% TFA) solvent, and the target mass was m/z 1200. The modification of the protein was followed by MS characterization using the charge state function of the compound(s) eluting with an absorbance at 410 nm. This method could clearly distinguish the signals of the native protein from those with one, two, or three R5P molecules attached (each adding a mass of 212 Da to the protein). Thus, the rates of glycation in the presence and absence of ATP can be compared.

Another technique was used to characterize the reverse effect, i.e., the effect of glycation on ATP binding.[5] Samples of cyt *c* (1.0 mg/mL) were incubated at 37 °C for 3 days with and without R5P (1.0 mM). Small aliquots (0.3 mL) of the solutions were placed onto a column loaded with ATP−agarose beads (C8-linked), and the sample was allowed to elute, first using Tris buffer (20 mM, pH 7.2) and then using 100 mM ATP (in Tris buffer). The amount of cyt *c* eluting from the column was estimated by the visible red color of the protein.

Spectral Change. The cyt *c* solutions incubated at 37 °C with or without R5P were combined with equal volumes of 10 mM HEPES (pH 7.4) and reduced with 0.3 mM ascorbic acid. The solutions (300 μL) were then microfiltered (14000*g* for 20 min) through 10000 MWCO filters followed by a 300 μL wash with 10 mM HEPES. The approximately 20 μL of solution remaining after the second centrifugation was retained (5000*g* for 5 min) and combined with 10 mM HEPES buffer to bring the concentration of cyt *c* back to 1.0 mg/mL. A 100 μL aliquot of this retentate was pipetted into a microwell, and the spectral data (a full spectrum, including the 410 nm isosbestic absorbance value and the 550 and 526 nm absorbance values) were collected using a Bio-Tek Synergy HT microplate reader. The 550 nm:526 nm absorbance ratio was used to determine the extent of reduction of the solutions based on known extinction coefficients.[24] All samples at this point proved to be in the fully reduced ferro-cyt *c* form. With the instrument held at 37 °C, HEPES buffer (10 mM) and either liposomes or prepared mitoplasts were added to bring the volume to 200 μL. The concentration of cyt *c* in the well was 0.5 mg/mL or 41 μM. Over 1−4 h, 550 and 526 nm absorbance values were measured every 15 s with shaking of the plate occurring prior to each measurement.

The analysis of changes at 550 nm versus 526 nm (an isosbestic point) is based on the change of ferro-cyt *c* from a low-spin state to a high-spin state upon liposome association. Thus, the 550 nm value will decrease, while the 526 nm value will remain the same during the binding event. The difference between the two absorbance values is used as a statement of the spin state change. (A ratio is not employed as different liposome amounts will raise or lower the overall baseline.) From the work of Kalanxhi and Wallace,[13] the spectral change value (a unitless value) at any point in time is calculated as

$$\text{spectral change} = [\,-(A_{550}-A_{526})_i - (A_{550}-A_{526})_o]/(A_{550}-A_{526})_o$$

In our experiments, the spectral change value would range from zero (no binding) to a plateau of 1.2 (associated with full cyt *c* spin state change). The time point at which the value crossed half-maximal change (i.e., 0.6) is defined as the $t_{1/2}$.

Liposome Binding Analysis by Microfiltration. Following the incubation of cyt *c* with liposomes or mitoplasts at 37 °C, the microwell samples were removed and microfiltered using 100000 MWCO filters (14000*g* for 15 min) to determine the amounts of free cyt *c*.[25] The percentage of free cyt *c* was determined by comparing the absorbance at 410 nm of the filtrate (100 μL) to the value obtained prior to adding the liposomes or mitoplasts to the well.

In these microfiltration studies, some loss of cyt *c* occurs, presumably as it binds to the microfilter membrane. On average, approximately 85% of free cyt *c* (i.e., not reacted with liposomes) subjected to our filtration method will enter the filtrate. Except for Figure 5, the data for the free cyt *c* were not corrected for this loss and are instead simply reported as a percentage of the starting solution.

Liposome Binding Analysis by Agarose Gel Electrophoresis. Cyt *c* samples (1 mg/mL), incubated for 24 h with or without R5P (10 mM), were combined at different ratios with unilamellar liposomes, subjected to agarose (0.4%) gel electrophoresis, and stained with Coomassie blue reagent.

Turbidity Changes. As one of our monitored wavelengths, i.e., 526 nm, was an isosbestic point of cyt *c*, we used this wavelength during the spectral change analysis as an indicator of turbidity change occurring during a reaction between cyt *c* and multilamellar liposomes. (The path length of the 200 μL in the microwell was 0.53 cm.) Upon completion of this analysis, representative samples were transferred for microscopic analysis. The typical magnification of the Zeiss inverted microscope was 200−400×.

Peroxidase Activity. The peroxidase activity of cyt *c* was measured using the 2,2′-azinobis(3-ethylbenzothiazoline-6-sulfonic acid) (ABTS)/H_2O_2 spectrophotometric procedure.[26] A cyt *c*-containing sample (25 μL) was added to 175 mL of ABTS (0.25 mg/mL) and H_2O_2 (0.005%) in 0.05 M citrate buffer (pH 6.0). The rate of increase in absorbance was monitored at 416 nm at room temperature. (The path length of the 200 μL in the microwell was 0.53 cm.)

RESULTS

Structural Changes Caused by Glycation of Cyt *c* by R5P. As shown by additions to the overall molecular mass of the protein by LC−MS analysis, the glycation of cyt *c* by R5P occurs at a relatively rapid rate. By 2 h at 37 °C in the presence of 10 mM R5P, a significant proportion (estimated to be 20%) of the protein was altered by the condensation of one R5P molecule as indicated by the addition of a group with a mass of 212 Da (Figure 1B compared to Figure 1A). This modification agrees with the initial proposed formation of a Schiff base and the subsequent Amadori product. (Both compounds are of the same

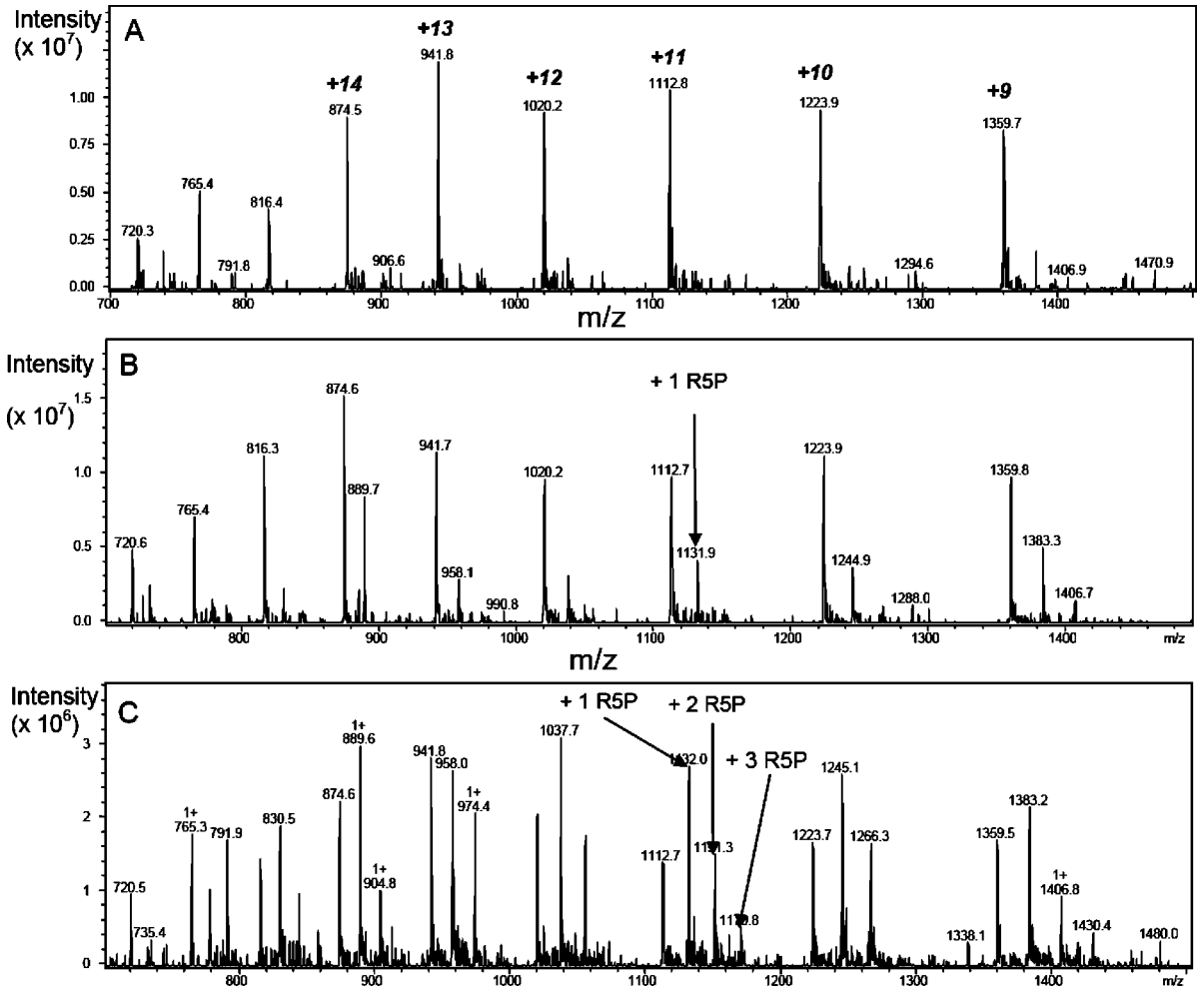

Figure 1. Reaction of R5P with bovine heart cyt *c* results in the attachment of two or three R5P molecules to the protein over an 8 h period. Each panel shows the various MS charge states of cyt *c* (indicated on spectrum A for the native protein) with a target mass of *m*/*z* 1200. The incubation times of the cyt *c* with R5P (10 mM) at 37 °C were 0 (A), 2 (B), and 8 h (C). The development of new spectral signals corresponding to the increased mass of the protein is indicated as an example for the +11 charged state form. Charge state analysis of these new signals revealed the addition of 212, 424, and 636 Da, which clearly represent the addition of one, two, and three R5P molecules, respectively.

mass; however, the Schiff base is a reversible product that would likely dissociate from the protein during the LC separation. The Amadori product is for all purposes an irreversible product.) MS signals corresponding to subsequent additions of a second and a third R5P molecule were also observed by 8 h (Figure 1C). With extended times (>20 h), the number of ion signals becomes so numerous that it was impossible to identify any one modified cyt *c* version as the predominate form. This indicates nearly complete modification of all cyt *c* molecules and various arrangements of R5P on the protein. We have previously shown that R5P undergoes dephosphorylation during its reaction with amines and that the series of subsequent arrangements can lead to multiple products.[17]

Evidence of modification of cyt *c* by R5P was supported by native gel electrophoresis, isoelectric focusing, and binding to DEAE-Sepharose beads. The addition of each R5P onto a surface Lys of cyt *c* results in a net −3 charge difference as each conjugation adds approximately two negative charges and covers up one positive charge. Native gel electrophoresis showed cyt *c* becoming increasingly more positive (see, for example, Figure 7) with increasing exposure to R5P, while isoelectric focusing analysis of this reaction resulted in a shift of a sharp band at pI = 10.5 to a broad band in the pI = 5.0−6.0 range. In addition, native cyt *c* exhibits tight binding to DEAE-Sepharose beads at pH 7, while less than 5% of the cyt *c* glycated by R5P (for 18 h) adhered to the beads.

The intensity of the Soret absorbance band failed to significantly change during reactions of cyt *c* (1 mg/mL) and R5P (50 mM) for periods of up to 7 days, indicating little heme damage. Measurement of the hydrogen peroxide levels in an aerobic reaction among R5P, Lys, and cyt *c* for 1 h indicated a H_2O_2 concentration of 70 μM. Thus, while the system produces H_2O_2 from the spontaneous dismutation of the superoxide generated by the reaction, cyt *c* resists the H_2O_2-promoted heme damage that is known to occur for myoglobin and hemoglobin.[27] This is likely due at least in part to the covalent attachment of the heme group to the cyt *c* chain. Hydrogen peroxide has also been shown to cause dimerization and oligomerization of cyt *c* over time.[28] SDS−PAGE analysis of a reaction between cyt *c* and R5P showed the protein becoming slightly larger (approximately 500−1000 Da) as sugars add to the protein; however, there was surprisingly no evidence of dimers or oligomers in R5P glycation reactions even at extended times (5 days).

Effect of ATP on Glycation. An LC−MS characterization of the glycation of cyt *c* (1 mg/mL) by R5P (10 mM) in the

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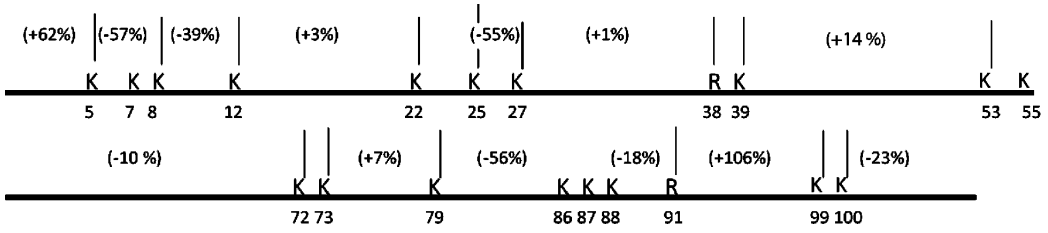

Figure 2. Representative schematic of the gain or loss of trypsin digest fragments upon R5P (10 mM) glycation of cyt *c* (1 mg/mL) for 3 days at 37 °C. The percentage shown for each fragment represents the gain (+) or loss (−) of that intensity of the EIC for that fragment vs that of a 0 h incubation control.

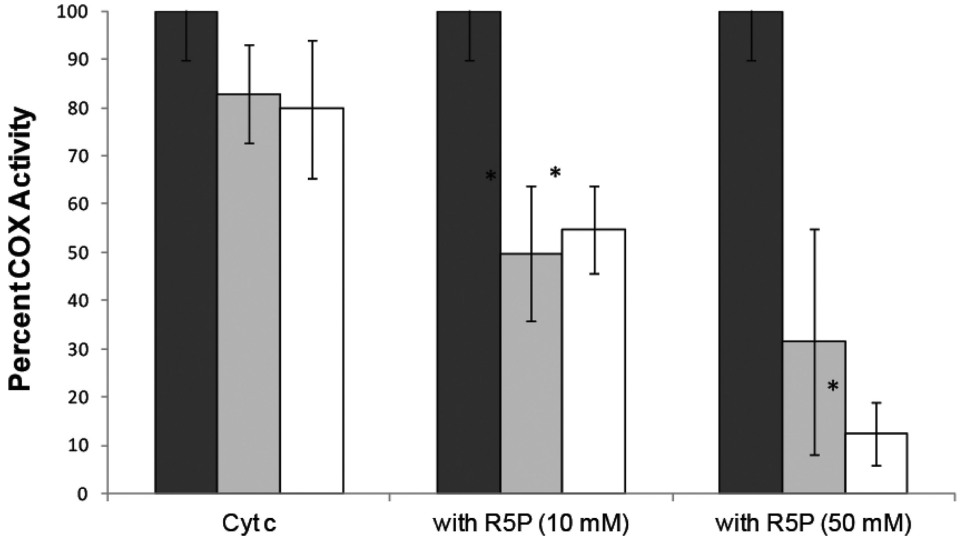

Figure 3. Glycation of cyt *c* (1 mg/mL) by R5P at 37 °C impairs its ability to transfer electrons to cytochrome oxidase. Units of COX activity were calculated in a spectrophotometric procedure employing isolated, soluble bovine heart COX and either untreated or R5P-glycated cyt *c* as a substrate. The values of four trials were normalized to the 0 h activity. The black bars show data for the 0 h incubation of cyt *c* with R5P, the gray bars data for the 6−8 h incubation, and the white bars data for the 20−24 h incubation. The error bars are ±1 standard deviation. Asterisks denote significance ($p < 0.5$) vs the untreated cyt *c* control.

presence of ATP (10 mM, room temperature) showed the slower development of a signal that correlated with R5P attachment than in the corresponding sample without ATP. After incubation for 5 h, the intensity of a signal for a population of cyt *c* molecules modified by one R5P molecule (mass of cyt *c* plus 212 Da) was approximately the same as that for the unmodified protein, while the corresponding signal for a monoglycated form was barely evident when the reaction was performed in the presence of ATP. We interpret this as an indication that ATP slows the rate of R5P glycation of cyt *c*.

The affinity of cyt *c* versus that R5P-glycated cyt *c* (1 mM, 3 days at 37 °C) for ATP conjugated onto agarose beads was characterized. While untreated cyt *c* adhered to the ATP−agarose beads when eluted with low-ionic strength buffer, a portion (estimated to be 50%) of the R5P-treated cyt *c* did not bind and instead eluted from the column. Addition of ATP to the ATP−agarose beads dislodged the bound cyt *c* in both cases. These results indicate that at least a portion of the cyt *c* molecules are modified at the ATP binding site in a manner that reduces the protein's affinity for the nucleotide.

Trypsin Fragmentation Studies. Analysis of the changes in the fragmentation profile created by trypsin cleavage of cyt *c* versus R5P-glycated cyt *c* was employed to determine the sites of glycation. This technique is based on the premise that trypsin will not cleave sites that have been modified by R5P. Thus, the relative amount of signal from fragments containing the altered Lys/Arg and the fragment immediately behind this site will be reduced in intensity with an increased level of modification. After reaction for 24 h, only Lys8 has significant levels of modification at approximately 35% (see the Supporting Information). As our observation of the overall mass changes indicates little unmodified protein at 24 h (see above), this means small amounts of glycation are occurring at various sites over the first 24 h. The results suggest that early in the reaction no one site seems to be a predominate site for glycation. Figure 2 displays the major fragments of cyt *c* and their decreases or increases in intensity upon R5P glycation for 3 days at 37 °C ($n = 6$). After reaction for 6 days, the results indicate that Lys8, Lys72, Lys87 or -88, and possibly Lys25 are the four sites modified by more than 70%. Several other sites have moderate levels of modification (see the Supporting Information). We searched the trypsin digestion for evidence of pieces with specific modifications but were unable to locate pieces of significant intensity with known glycation forms (such as the R5P-attached form or the carboxymethyl lysine form).

Cyt *c* Function in the Respiratory Chain. The ability of R5P-glycated cyt *c* to transfer electrons to COX was measured spectrophotometrically using an isolated, soluble COX preparation. Cyt *c* incubated at 37 °C with or without R5P (50 or 10 mM) was, following complete reduction, added to COX and the rate of oxidation of cyt *c* monitored. Figure 3 shows the activity results normalized to the 0 h incubation

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activity. (This was done because different activities of COX were used in some of the experiments.) Incubated for 24 h at 37 °C, the untreated cyt *c* retained approximately 80% of its original capacity to transfer of electrons to COX, while the cyt *c* treated with 10 and 50 mM R5P showed significantly ($p < 0.05$) less ability to transfer electrons, only 55 and 12%, respectively. (After incubation for 6−8 h, the protein treated with 50 mM R5P showed a significant difference vs the control while the cyt *c* treated with 10 mM R5P did not.) A similar trial using glucose 6-phosphate over a 3 day period did not show any significant difference versus the cyt *c* control. We observed no effect of R5P on COX itself.

Membrane Association of Glycated Cyt *c*. The ability of glycated cyt *c* to interact with cardiolipin-containing membranes was assessed by several methods. First, washed ferro-cyt *c* samples reacted with R5P for 2.5−18 h at 37 °C were subsequently incubated with cardiolipin-containing unilamellar liposomes for 1−4 h at 37 °C and then microfiltered through a 100000 MWCO filter that retains the liposomes and anything that associates with them.[25] The association was dependent on the ionic strength of the medium, with stronger association occurring at the lower ionic strengths provided by the 10 mM HEPES buffer.[29] Table 1 illustrates the

Table 1. Spectral Changes and Liposome Binding[a]

incubation time (h)	bovine cyt *c* (1 mg/mL)	binding agent (ratio)[b]	spectral change $t_{1/2}$ (min)	binding free cyt *c* (%)
2.5	untreated	liposomes (1:5) (unilamellar)	92 ± 5	6.8 ± 0.5
	treated with R5P (10 mM)		124 ± 8	7.1 ± 0.3
18	untreated	liposomes (1:5) (unilamellar)	86 ± 6	44 ± 4
	treated with R5P (10 mM)		145 ± 2	73 ± 4
	treated with R5P (10 mM) and ATP (10 mM)		101 ± 4	51 ± 2
24	untreated	liposomes (1:5) (multilamellar)	23 ± 6	23 ± 13
	treated with R5P (10 mM)		47 ± 6	71 ± 21
	treated with R5P (10 mM) and ATP (10 mM)		21 ± 1	30 ± 1
24	untreated	mitoblasts (1.4:1)	−	12 ± 5
	treated with R5P (10 mM)		−	63 ± 6
	treated with yeast cyt *c* (1 mg/mL)			
18	untreated	liposomes (1:1) (unilamellar)	12	37
	treated with R5P (10 mM)		54	49
	treated with R5P (10 mM) and ATP (10 mM)		8	37

[a]All error ranges given are ±1 standard deviation. [b]For liposomes, molar ratios of cyt *c* to total lipid are given. For mitoblasts, ratios are ratios of cyt *c* to mitochondria. Binding values are for a 1−4 h incubation at 37 °C.

percentage of the total cyt *c* found in the filtrate, i.e., that which did not bind the liposomes. The percent cyt *c* found in the filtrate was not significantly different for the cyt *c* treated with R5P for 2.5 h and untreated cyt *c*, while weakened binding (approximately 50%) was observed for the treated cyt *c* when a longer R5P glycation time of 18 h was employed. We obtained similar results for experiments using large, multilamellar liposomes (Table 1), except significant differences in binding were observed for the 2.5 h incubation samples and overall the larger liposomes had a stronger binding nature. The differences in the association of glycated versus unglycated cyt *c* to cardiolipin-containing liposomes were also seen at other liposome:cyt *c* ratios (1:2 to 1:20), with an expected stronger binding occurring with higher liposome concentrations.

If one assumes a 1:1 binding stoichiometry between cyt *c* and the cardiolipin contained in the liposomes, an estimated equilibrium constant for binding (K_B) of 4×10^5 M^{-1} is obtained. The K_B for cyt *c* held for 18 h was lower, presumably because of some cyt *c* denaturation. Comparatively, the binding constant for cyt *c* is approximately 8-fold greater than that of the R5P-glycated cyt *c*. Although the relationship of binding of cyt *c* to liposome components has yet to be clearly defined, it is worth noting that the number of cyt *c* molecules bound to the liposomes in some cases (i.e., at high cyt *c*:cardiolipin ratios) exceeds the 1:2 stoichiometric ratio suggested to be involved in permanently holding the cyt *c* to the liposomes.

The glycation reaction of cyt *c* with R5P (10 mM) performed in the presence of an equimolar amount of ATP caused the affinity of the resultant cyt *c* for unilamellar liposomes to increase (Table 1). In experiments using multilamellar liposomes, equimolar ATP completely reversed the loss of binding affinity to that of the untreated cyt *c*. These results suggest ATP is protecting an important liposome binding site from R5P glycation.

Yeast cyt *c* (mainly iso-1-cyt *c*) showed a significantly stronger tendency to bind to liposomes than bovine cyt *c*. (To more accurately detect these changes, we reduced the amount of liposomes to a ratio of 1:1.) Glycation of yeast cyt *c* for 18 h similarly weakened the ability of the protein to bind to the unilamellar liposomes (Table 1). In the same manner as bovine cyt *c*, the presence of ATP during the incubation of cyt *c* with R5P strengthened the tendency of yeast cyt *c* to bind to liposomes.

Binding of R5P-glycated cyt *c* to prepared mitoplasts was similarly impaired (Table 1) in comparison to that of the nonglycated protein. When the relative amount of mitoplast was varied in the incubation mixture, the nonglycated cyt *c* showed the expected change in binding (from 35% bound at the lowest mitoplast level to 85% bound for the highest), suggestive of tight binding to a limited amount of binding sites on the membrane. The amount of R5P-glycated cyt *c* bound to mitoblast, however, remained relatively constant (at 30−40%) during this variation in the amount of mitoplasts. This indicates that a population of cyt *c* molecules was altered sufficiently by the glycation to render them incapable of mitoplast binding. It is important to note that during this process, the cyt *c* was rapidly oxidized (presumably by its interaction with active cytochrome oxidase), and thus, the interaction observed was likely a ferri-cyt *c*−membrane association.

Characterizing the association of cyt *c* with cardiolipin-containing liposomes can also be assessed by observing the decrease in absorbance at 550 nm (vs 526 nm) of reduced cyt *c* as the protein binds to the membrane.[13] The "spectral change" as the α and β peaks coalesce is shown in Figure 4 for an unglycated sample (reduced prior to the spectroscopic analysis) and for a R5P-glycated sample (24 h at 37 °C) using multilamellar

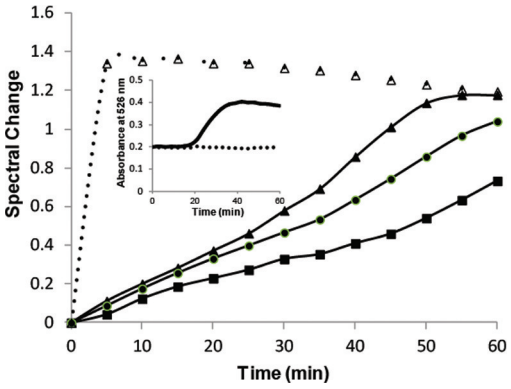

Figure 4. R5P glycation slows the spectral change of cyt *c* upon its incubation with cardiolipin-containing multilamellar liposomes. The rate of spectral change [$\Delta A_{550} - A_{526}$ (see the text)] is shown upon the reaction of cyt *c* (0.5 mg/mL) with liposomes (molar ratio of 1:5) at 37 °C for 1 h. Pretreatment of cyt *c* (37 °C for 24 h): untreated bovine cyt *c* (▲), bovine cyt *c* incubated with R5P (10 mM) (■), and bovine cyt *c* incubated with R5P (10 mM) and ATP (0.5 mM) (●). For comparison, the spectral change of unglycated yeast cyt *c* (37 °C for 24 h) (△) is shown. In the inset, the A_{526} curves for untreated bovine cyt *c* (thick line) and R5P-glycated bovine cyt *c* (●) mixed with multilamellar liposomes show turbidity changes for the untreated cyt *c* that are not observed for the glycated protein.

liposomes (1:5 ratio). We observed the expected decrease in $t_{1/2}$ as we moved from lower (1:2) to higher (1:20) liposome ratios. As shown in Table 1, the rate of spectral change for the glycated samples is significantly slower than for the unglycated samples. For 2.5 h incubation samples, the $t_{1/2}$ for the untreated samples mixed in a 1:5 ratio with unilamellar liposomes was 92 min while that for the R5P-glycated samples was 124 min. Extending the incubation time with R5P further slowed the association of the glycated cyt *c* with liposomes. After reaction for 18 h, the $t_{1/2}$ of R5P-glycated cyt *c* was 145 min, approximately double the $t_{1/2}$ of the unglycated samples. Interestingly, the comparative spectral change for reactions using multilamellar liposomes was faster despite a smaller available surface area.

The simultaneous treatment of R5P (10 mM) and ATP (10 mM) with bovine cyt *c* reversed the trend of longer $t_{1/2}$ times partially (unilamellar) and fully (multilamellar) (Table 1 and Figure 4). ATP placed directly in the reaction mixture of untreated ferro-cyt *c* and liposomes caused a slowing of the rate of spectral change (2-fold change in $t_{1/2}$ using multilamellar liposomes) that was equal to that caused by simply increasing the ionic strength using NaCl. Thus, the role of ATP in limiting the effect of R5P glycation on cyt *c*'s liposome binding is in opposition to the effect normally seen for ATP on the association of untreated cyt *c* to membranes.

In agreement with our binding assessment conducted via microfiltration, the rate of spectral change using yeast cyt *c* and phosphatidylcholine/cardiolipin liposomes was significantly faster than with bovine cyt *c* (Figure 4 and Table 1). R5P glycation of yeast cyt *c* similarly caused longer $t_{1/2}$ times, and inclusion of ATP in the reaction reversed this effect.

The spectral change profiles did not appear to be consistent with the data we obtained from our microfiltration binding studies. For example, although the spectral change in many cases had reached 100% of cyt *c* molecules undergoing spin state change, thus suggesting acyl chain insertion, we still observed free cyt *c* in solution. To understand this process more fully, we generated a spectral change profile while gathering liposome binding percentages (via microfiltration) at the shorter time intervals. The two processes appear to be linked as the timing of the spectral change increase synchronized with the values of percent bound (Figure 5).

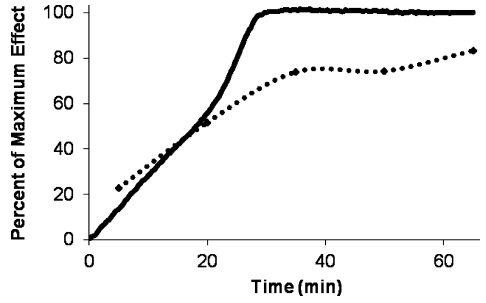

Figure 5. Spectral change correlates with binding (microfiltration analysis) with incubation time for interaction of bovine cyt *c* (0.5 mg/mL) with multilamellar liposomes (1:5 molar ratio) but does not agree with the extent of binding at longer incubation times. Both graphs show "association" on a percentage scale of the maximal observed effect. The spectral change (thick line) and the binding percentage (◆) both increase from 0 to 60% on the same time scale, but while the spectral change increases to 100%, the microfiltration level at this cyt *c*:liposome ratio is only 80%.

However, at the end of incubation at 37 °C for 1 h with multilamellar liposomes, the point at which we saw complete spectral change indicating a full shift to the high-spin form, we observed a portion of the free cyt *c* moving in the filtrate. Spectral analysis (A_{550}/A_{526} ratio) of the free cyt *c* that separated from the liposome fraction into the filtrate indicated the protein to be in a high-spin state.

The A_{550} and A_{526} absorbance curves themselves varied between trials when using multilamellar liposomes (1:5 ratio). While all exhibited the expected decreasing difference profile between the A_{550} and A_{526} values of cyt *c* upon incubation with the liposomes (see Figure 4), the samples not treated with R5P gave A_{526} curves (inset of Figure 4) that remained constant at first but then increased. (A similar increase was seen in the 550 nm absorbance, albeit not as fast.) The increase in A_{526} was not observed for samples treated with R5P, with or without ATP. Instead, the interaction of glycated cyt *c* with liposomes showed an A_{526} curve that simply held constant in absorbance (see the inset of Figure 4). The increase in the A_{526} curves for unglycated cyt *c* samples was associated with a significant increase in the observable turbidity of these samples.[12] Visibly, it appeared the liposomes were coalescing and defracting more light. This was confirmed by microscopic analysis (Figure 6) showing the multilamellar liposomes themselves (without reaction with cyt *c*) to be relatively small [up to 15 μm (Figure 6A)]; the liposomes reacted with glycated cyt *c* and were somewhat larger [up to 30 μm (Figure 6C)] but still spherical, and the liposomes reacted with untreated cyt *c* to form large lipid aggregates [up to approximately 100 μm (Figure 6B)], which seemed to be comprised of small liposomes clumped together. ATP (4 mM) added at the start of the liposome-unglycated cytochrome incubation inhibited the increase in turbidity as did an addition of NaCl (12 mM).

When mitoplasts were used, the solutions similarly became turbid when using untreated cyt *c* but not glycated cyt *c*. Under magnification, the mitoplasts exposed to untreated cyt *c* were

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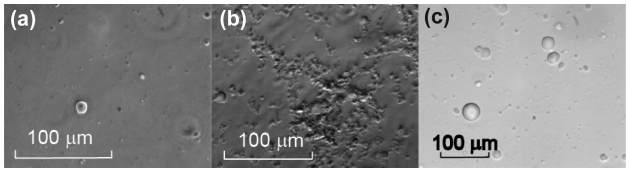

Figure 6. Microscopic analysis of cytochrome–liposome interaction shows that R5P glycation of cyt *c* reduces the protein's tendency to form large aggregates. (A) Multilamellar liposomes untreated with cyt *c*. The liposomes were approximately 10 μm in diameter on average. (B) Multilamellar liposomes treated with untreated cyt *c* for 1 h at 37 °C. The aggregate in the center of the picture is approximately 100 μm in diameter. (C) Multilamellar liposomes treated with R5P-glycated cyt *c* for 1 h at 37 °C. The typical diameter of the individual liposomes is approximately 35 μm.

approximately 2-fold larger on average than those exposed to glycated cyt *c*, which were on average 3-fold larger than unexposed mitoplasts (see the Supporting Information).

A similar set of experiments (microfiltration and spectral change to liposomes) were performed using D-glucose as the glycating sugar. After reaction for 1 week at 37 °C, glucose-glycated cyt *c* incubated with multilamellar liposomes (1:5) showed little change in binding versus the control, with a value of 7.6% free cyt *c*, while the unglycated sample yielded 6.0% free cyt *c*. The spectral changes at 550 nm were only slightly slower for the treated samples than the untreated samples. The $t_{1/2}$ for the glucose-glycated samples combined 1:5 with liposomes was 10 min, while that for the untreated sample was 11 min. In total, these data suggest the modification of cyt *c* with glucose for 1 week at 37 °C was relatively insignificant.

A third technique for assessing association of cyt *c* with a liposome membrane is to monitor the effect liposomes have on the migration of cyt *c* during agarose gel electrophoresis.[30] The highly positive (+8 at neutral pH) cyt *c* migrates toward the cathode unless it is bound to the negatively charged liposomes. When the ratio of liposomes to cyt *c* increases, more cyt *c* is attached and the membrane-bound cyt *c* is directed toward the anode (Figure 7). Untreated cyt *c* has been shown to shift its movement toward the anode at cardiolipin ratios of approximately 1:4 and higher.[30] In our experiment, we see clear movement of cyt *c* at a 1:4 cyt *c*:total lipid ratio (Figure 7, lane 5). As mentioned previously, glycation of cyt *c* by R5P makes the protein more negatively charged, and it migrates more rapidly toward the anode (Figure 7, lane 7 vs lane 8). Except for a small population of cyt *c*, treatment of glycated cyt *c* (18 h at 37 °C) with unilamellar liposomes did not significantly change the protein's electrophoretic mobility (Figure 7, lanes 1–3 vs lane 7), while a significant change in mobility was observed for untreated cyt *c* (Figure 7, lanes 4–6 vs lane 8). Thus, treatment of cyt *c* with R5P appears to hinder the association of cyt *c* for membranes.

Cyt *c* Peroxidase Activity. The peroxidase activity of R5P-glycated (1 day at 37 °C) ferro-cyt *c* did not change significantly (initial rate at 416 nm, ~0.175 A/min) from that of the untreated sample. After incubation for 6 days at 37 °C, however, the peroxidase activity of the R5P-treated ferro-cyt *c* was approximately 50% greater than that of the untreated ferro-cyt *c*. As expected, we saw increased activity (approximately 2-fold in both cases) for these samples after they had reacted with liposomes.

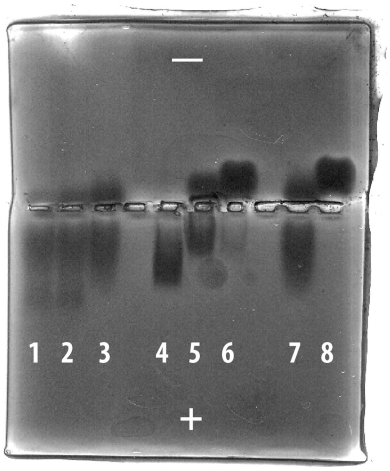

Figure 7. R5P glycation weakens the association of bovine cyt *c* with cardiolipin-containing liposomes. Agarose gel electrophoresis of R5P-treated or untreated cyt *c* incubated with unilamellar liposomes at different ratios for 1 h at 37 °C: lanes 1–3, R5P-treated cyt *c* and liposomes at 1:8, 1:4, and 1:2 ratios, respectively; lanes 4–6, untreated cyt *c* and liposomes at 1:8, 1:4, and 1:2 ratios, respectively; lane 7, R5P-treated cyt *c* without liposome treatment; lane 8, untreated cyt *c* without liposome treatment. The R5P glycation reaction of cyt *c* was conducted for 18 h at 37 °C. Samples were reduced and microfiltered prior to electrophoresis.

DISCUSSION

Cyt *c* plays two major functions in the cell: respiratory transport and apoptosis initiation. The proper function of cyt *c* in both of these processes requires surface lysines for interaction with its binding partner. In the reaction with cytochrome oxidase, a ring of lysines (Lys8, -13, -27, -72, and -87)[3] that encircle the heme edge of cyt *c* electrostatically bond to oppositely charged residues on the oxidase, allowing contact of the heme groups and subsequent transfer of electrons.[1,31] In its interaction with cytochrome reductase, two critical lysines, Lys13 and Lys86, were originally suggested to be involved[32] while nonpolar residues were later shown to be important.[2] In binding to mitochondrial membranes, specific lysines at the A-site or perhaps neighboring the C-site interact with the membrane's cardiolipin to encourage insertion of an acyl chain at one or two cavities within the cyt *c* interior.[12,14,29] Additionally, cyt *c* also houses an ATP binding site that includes a group of lysines (Lys72, -86, and -87) and a critical arginine, Arg91.[4,33] ATP binding to this site has been thought to coordinate lysines and/or Arg91 needed by cyt *c* for interaction with respiratory chain partners and with the cardiolipin-containing mitochondrial membrane.[5,14,34,35] Spontaneous glycation of any of the important surface basic residues will cover the positive charges needed for interaction, thereby weakening the ability of cyt *c* to function properly. This effect may be even more dramatic if the glycation agent is a negatively charged R5P molecule as opposed to a neutral sugar like glucose.

It is evident from our studies that association of R5P with and glycation of cyt *c* affect the protein's function in respiration and pro-apoptosis. Previously, we have shown that R5P at millimolar levels can rapidly reduce ferri-cyt *c*, generating O_2^- in the process.[18] The observation that the reduction rate can be slowed by ATP suggests that a primary site of interaction of R5P on cyt *c* is this site or at least involves some of the ATP-interacting residues, including Lys86, Lys87, Lys88, Arg91, or

perhaps Lys72 and Lys73. The results of our experiments showing the lack of ATP−agarose affinity of a subset of cyt *c* molecules glycated by R5P are in agreement with the suggestion that R5P targets some of the same lysine residues that are responsible for ATP binding, respiratory chain partner association, and membrane association. On the same time frame, our LC−MS studies of the overall modification of cyt *c* by R5P indicate that these two molecules readily react, forming Schiff bases, Amadori products, and advanced glycation products. Our trypsin fragmentation analysis of glycation sites implicates many of the lysines involved in the electron transport and membrane binding processes.

Two main conclusions can be drawn from the trypsin digestion analysis of unglycated versus R5P-glycated cyt *c*. The first is that there is no single target of glycation on cyt *c*. Almost all fragments terminated with lysines showed decreases in signal intensity with extended lengths of R5P glycation. This is expected as the pK_a values of the various lysines are likely not significantly different, which would allow one site to dominate as the glycation site. Conversely, it does appear that there are some favored sites and that Lys87/88 and Lys72 are among these. Modification of these residues, important for ATP binding, respiratory chain partner docking, and membrane association, is obviously detrimental to the function of the protein.

In its normal mitochondrial environment, cyt *c* exists primarily as a free or loosely bound protein with approximately one-eighth of the total protein being tightly bound to the membrane. The interaction of cyt *c* with cardiolipin-containing membranes is thought to be at one of two sites, the A-site involving Lys72, Lys73, Lys86, Lys87, and Arg91 and the C-site that involves Asn52.[36] Kalanxhi and Wallace[13] showed that Lys72 and Arg91 were crucial to the binding of cyt *c* to the membrane surface. Insertion of an acyl chain upon binding of the A-site was suggested to pass through a slot that includes hydrophobic regions around residues 67−71 and 82−85. Sinibaldi et al.[15] more recently have proposed a double-acyl chain insertion with the second chain penetrating near Asn52, Ile35, Trp59, Met64, Tyr67, and Ile75.[14]

The spectral change at 550 nm is not due to the oxidation of the reduced cyt *c* but instead is a low-to-high-spin state change occurring when a fatty acid acyl chain inserts into the protein, thereby disrupting the Met80 coordination.[9,13,25,37] Kalanxhi and Wallace[13] suggest a two-step process with the initial step being the electrostatic interaction between positive groups on cyt *c* and the negative cardiolipin and the second step being the slower penetration of the acyl chain into the cyt *c* protein. Their results suggest that electrostatic interactions are important not only for initial association but also for maintaining acyl chain insertion. In other words, the acyl chain insertion is not necessarily a permanent state. They also observed binding and the low-to-high-spin transition in membranes not containing cardiolipin. In Asn52 mutant studies, Sinibaldi et al.[15] noted a biphasic binding process suggestive of two acyl chain insertions. Thus, a reaction sequence for the strong binding of cyt *c* to a membrane surface involves more than a single rate constant for the electrostatic association (i.e., rate constants for cardiolipin binding at the A-site and C-site along with non-cardiolipin bonding) and more than a single rate constant for acyl chain insertion (i.e., A-site and C-site). Their proposal is that two acyl chains of a single cardiolipin molecule insert into the cyt *c* molecule (one at the A-site and the other at the C-site) while the other two chains of cardiolipin remain fixed to the liposome. In a study that characterized the increased turbidity that occurs when cyt *c* is incubated with liposomes, Kawai et al.[12] also concluded that two different sites on the cyt *c* interacted with liposomes and that this double interaction on opposite sides of the protein caused liposomes to fuse. They, however, concluded the two sites were the L-site (involving Lys22 and Lys27) and the A-site (involving Lys72 and Lys73). Carbethoxylation of specific groups (Lys22, Lys27, His33, and Lys87) of cyt *c* was shown to prevent vesicle fusion but did not stop the association of the heme protein with the membrane.[12]

The spectral studies and the binding studies confirm the work of others in showing that ferro-cyt *c* associates with cardiolipin-containing liposomes. As expected, the speed of the spectral change is increased with increasing liposome-to-cyt *c* molar ratios, and the percent of cyt *c* bound to liposomes (microfiltration studies) for 1 h at 37 °C is likewise greater for the higher ratios. From these and previous results, we originally assumed that both the spectral change study and the microfiltration binding study are detecting those cyt *c* molecules that have acyl chain insertion. However, our microfiltration binding studies clearly show a difference in cyt *c*−multilamellar liposome binding between the 1:8 and 1:20 ratios after reaction for 1 h, while the spectral change results indicate that all of the cyt *c* molecules have shifted from the low-spin form to the high-spin form, thus implying all protein has undergone acyl chain insertion. Thus, either the acyl chain insertion event is a reversible process, or the spin state shift caused by the movement of the methionine does not necessarily coincide with the permanent acyl chain penetration. Balakrishnan et al.[38] have shown that the spin state change of cyt *c* can also occur at low pH (pH ∼3) and moderately high temperatures (>50 °C) as the protein shifts to a β-sheet form. This is accompanied by a corresponding increase in the peroxidase activity of cyt *c*. Balakrishnan et al. speculate that this shift could happen upon association of cyt *c* onto a cardiolipin-containing membrane and that this conformational change, rather than acyl chain insertion, could be the initial step in the pro-apoptosis process. If this association is readily reversible, a spectral change would be observed while the binding levels would not show permanent binding until a slower acyl chain insertion occurs. This would imply a two-step process in which the initial association of cyt *c* onto the cardiolipin-containing membrane results in a change in conformation (to the β-sheet form) and then the associated cyt *c* would undergo the acyl chain insertion and permanent binding at the A-site, the C-site, or both.

Yeast cyt *c* demonstrated a faster spectral change and a tighter association with cardiolipin-containing liposomes than bovine cyt *c*. (This is in contrast with the results Kalanxhi and Wallace[13] that showed greater binding affinity for the mammalian cyt *c*.) Similar to that of the bovine form, glycation of yeast cyt *c* increased the $t_{1/2}$ of the spectral change and decreased the binding affinity of the protein for membranes. The ability of ATP to reverse the R5P effects on yeast cyt *c* is interpreted as ATP preventing R5P glycation at the A-site of yeast cyt *c*, thus leading to improved liposome binding. In this case, however, the glycation site affected cannot be Lys72 as the site on yeast iso-1-cyt *c* is trimethylated and unreactive to glycation. Interestingly, we did not observe the turbidity increase in untreated yeast cyt *c* when it reacted with cardiolipin-containing multilamellar liposomes.

Glycation affects membrane association. The spectral studies and the binding studies are in agreement in showing that a ≥2.5 h glycation with R5P inhibits cyt *c*−liposome association.

The spectral studies likely record only the insertion at the A-site, whereas the microfiltration studies would determine the combined binding at either site. Our trypsin digestion studies indicate that Lys72 and Lys87/88, critical lysines at the A site, are glycated at higher rates than other lysines of the protein. Our ATP competition studies further support one or both of these as targets for R5P interaction. Modification of these residues with R5P or R5P-generated advanced glycation end products would eliminate the positive charge needed for the initial electrostatic interaction and/or may cause steric difficulties for the cyt *c* in closely interacting with the cardiolipin membrane groups. In any case, it appears from our results that the modifications we observe with short R5P incubation times (<24 h) are not sufficient to prevent the actual association and acyl chain insertion.

Kawai et al.[12] observed a turbidity increase when cardiolipin-containing liposomes were incubated with cyt *c* and proposed the increase in light scattering was due to the attachment of separate liposomes to two sites of a cyt *c* molecule (A-site and L-site) and then a subsequent membrane fusion to create larger vesicles. The failure to observe a change in turbidity for the reaction of R5P-glycated cyt *c* with multilamellar liposomes while we clearly see spectral change and some liposome binding suggests that our treatment is primarily affecting one of two cardiolipin binding sites. The most likely candidate for this site is Lys72. As one of the lysines of the A-site thought to be involved in cardiolipin association, Lys72 was shown to be one of the primary targets of R5P glycation by our trypsin digestion studies. Moreover, the failure of trimethylated Lys-containing yeast cyt *c* to exhibit similar turbidity changes suggests a role of this residue during the membrane fusion process.

The fact we did not observe similar modification reactions of bovine cyt *c* with glucose during a 1 week incubation emphasizes the preference for R5P to specifically target a site that is not readily recognized by glucose. We suggest this is due to the intramolecular phosphate of R5P that directs it to a highly positive site on the surface of cyt *c*. The neutral (and highly unreactive) glucose is not attracted to this site and, hence, fails to show significant inhibition of the cyt *c*–liposome interaction.

In summary, R5P glycation appears to greatly affect cyt *c*'s function in both the respiratory chain and pro-apoptotic events. Along with its intriguing ability to rapidly reduce cyt *c*, the sugar phosphate attaches readily to the heme protein to cover critical lysines needed for its association with the negative surfaces of its respiratory chain partners and of cardiolipin contained in the inner membrane. Evidence strongly suggests that the R5P glycation of critical residues at the A-site causes a reduction in the affinity of cyt *c* for liposome surfaces and that this then inhibits the tendency for liposomes to undergo fusion, an early step in the apoptotic reaction. The results indicate that control of the R5P level in cells could play a role in important cyt *c*-mediated processes in vivo.

■ ASSOCIATED CONTENT

Supporting Information

LC–MS analysis of trypsin digestion of glycated cyt *c* and criteria for designating glycation sites. This material is available free of charge via the Internet at http://pubs.acs.org.

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■ ABBREVIATIONS

ABTS, 2,2′-azinobis(3-ethylbenzothiazoline-6-sulfonic acid); cyt *c*, cytochrome *c*; COX, cytochrome oxidase; dR5P, 2-deoxyribose 5-phosphate; R5P, ribose 5-phosphate.

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