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

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Abstract

Spontaneous glycation of bovine heart cytochrome c (cyt c) by the sugar ribose 5-phosphate (R5P) decreases 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 decreased its ability to transfer electrons to cytochrome oxidase by 60%. An 18 hour treatment with R5P decreased bovine cyt c's binding affinity with cardiolipin-containing liposomes by an estimated eightfold. A similar lower 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 Met coordination to heme, was found for the R5P-treated cyt c. This change occurred to a greater extent than explained by the permanent attachment of the protein onto the liposome. Turbidity changes resulting from the multi-lamellar 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.

Keywords

Ribose 5-phosphate; cytochrome *c*; cardiolipin

As the only non-integral 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 enables the heme-to-heme contact needed for efficient electron transfer (1, 2, 3). Important lysines, such as Lys87, that function in this patch can be shifted in position 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, 6, 7).

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SUPPORTING INFORMATION AVAILABLE: LC-MS analysis of trypsin digestion of glycated cyt c; criteria for designating glycation sites. The material is available free of charge via Internet at http://pubs.acs.org.

Cyt c also has an attraction to the outer leaflet of the inner mitochondrial membrane (8, 9, 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) which 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 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 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 cyt c exit from the inter membrane 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 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. Due to its relatively high 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 of approximately 100 times that of relatively non-reactive sugars such as glucose (16, 17). Containing a negatively charged phosphate group, R5P may have particularly high reactivity towards 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* has great importance as increased 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 be increased in concentration 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 a reduced ability of cyt c to transfer electrons to cytochrome oxidase. In addition, the affinity of R5P-glycated cyt c to cardiolipin-containing liposomes and to 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 d. 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 two 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 550 nm absorbance was monitored over two minutes at room temperature. One Unit of COX activity is defined as the ability of the enzyme to convert 1 μmol reduced cyt c ($\Delta\varepsilon_{550}$ = 19.6 mM^{-1} cm^{-1}) to the oxidized form per minute of reaction. Results were analyzed by a Student t-test.

Cyt c glycation analysis by LC-MS

The attachment of R5P molecules onto bovine heart cyt c can be monitored with reaction time by liquid chromatography-mass spectrometry (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 carried out using a 1:1 water:acetonitrile (both with 0.1 % trifluoroacetic acid, TFA). The compounds eluting from the LC were analyzed by ion trap MS using a target *m/z* = 1500. The molecular mass of the cyt c products were determined by charge state analysis

Cyt c trypsin digestion

LC-MS analysis of tryptic digests was carried out on untreated cyt c and on cyt c reacted with R5P (10 mM) at 37 °C for up to six days. Cyt c solutions denatured in 6 M urea for one hour at 50 °C were microfiltered through Amicon Microcon® 10,000 molecular weight cut-off (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 allowed to incubate for 16 – 20 h at 37 °C. All samples were analyzed using the Agilent ion trap LC-MS with a Zorbax C18 reverse phase column. Gradient elution (0.5 mL/min) of the digested pieces was accomplished starting at 100 % water and rising linearly to 70 % acetonitrile over a 50 min period. (Both solutions contained 0.1 % TFA.) The target mass was set at an *m/z* = 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 were present (i.e., Lys6-Lys7; Arg38-Lys39; Lys72-Lys73; Lys86-Lys-87-Lys88; 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 at 0 h of R5P incubation to evaluate the decreased trypsin action at the site due to glycation modification. Criteria were established for the designating a particular lysine or arginine as a highly probable target of R5P

glycation. (See Supplemental 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 2.0 mg/mL concentrations 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 (about 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 (approximately 2 minutes). Unless noted, a 1680 μM total lipid solution was prepared with a concentration of 20 % cardiolipin. These large multi-lamellar vesicles (estimated in size to be 0.5 – 15 μm) were used for some binding experiments (noted below as “multi-lamellar”). Most of the spectral change experiments were performed using a uni-lamellar liposome preparation (13). The multi-lamellar solution was subjected to five cycles of a rapid freeze-thaw followed by vortexing for one minute. This solution was then filtered thirteen times through a 0.1 μm polycarbonate membrane at 37 °C using a mini-extruder (Avanti Polar Lipids, Inc.). This treatment gives uni-lamellar liposomes (termed below as “uni-lamellar”) of approximately 100 μm in diameter. Uni-lamellar 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

Mitoplasts were prepared (6) by homogenizing bovine heart tissue with a Potter-Elvehjem at 4 °C in a ten-fold volume of buffer (250 mM sucrose, 10 mM Tris, 2 mM EDTA, pH 7.2), centrifuging at 3000 × g to pellet the nuclei and membranes, and centrifuging the subsequent supernatant at 12,000 × g to pellet the mitochondria. The outer membrane was ruptured and the loosely bound cyt c was dislodged by subsequent treatments with 15 mM KCl and 150 mM KCl (with 12,000 × g centrifugations). The final mitoplast preparation (in 10 mM KCl) was stored at 4 °C until use.

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 up to five hours. 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 daltons to the protein). Thus the rate of glycation in the presence or 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 three 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 with 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 (14,000 × g; 20 min) through 10,000 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; 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 isobestic 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 nm 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 vs. 526 nm (an isobestic point) is based on the change of ferro-cyt c from a low spin 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 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 where 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 100,000 MWCO filters (14,000 × g; 15 min) to determine free cyt c amounts (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/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 was not corrected for this loss and is 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 uni-lamellar 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 isobestic 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 multi-lamellar liposomes. (The pathlength 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 – 400X.

Peroxidase Activity

The peroxidase activity of cyt c was measured using the 2,2′-Azino-bis(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 pathlength of the 200 μL in the microwell was 0.53 cm.)

RESULTS

Glycation of Cyt c by R5P – Structural Changes

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 at 20%) of the protein was altered by the condensation of one R5P molecule as indicated by the addition of a group with mass 212 daltons (Fig 1B compared to the Fig 1A). This modification agrees with the initial proposed formation of a Schiff base and the subsequent Amadori product. (Both compounds are of the same mass, however the Schiff base is a reversible product that would likely dissociate off 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 (Fig 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 studies 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 2 negatively charges and covers up one positive charge. Native gel electrophoresis showed cyt c becoming increasing more positive (see for example Fig. 7) with time of 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 up to seven days, indicating little heme damage. Measurement of the hydrogen peroxide levels in an aerobic reaction between 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 with time (28). SDS PAGE analysis of a reaction between cyt c and R5P showed the protein becoming slightly larger in mass (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).

ATP Effect on Glycation

An LC-MS characterization of the glycation of cyt c (1 mg/mL) by R5P (10 mM) in the presence of ATP (10 mM, room temperature) showed a slower development of a signal that correlated with R5P attachment than the corresponding sample without ATP. At five hours of incubation, a signal for a population of cyt c molecules modified by one R5P molecule (mass of cyt c plus 212 daltons) was approximately the same intensity as that for the unmodified protein while the corresponding signal for a mono-glycated form was barely evident when the reaction was performed in the presence of ATP. We interpret this as indicating that ATP slows the rate of R5P glycation of cyt c.

The affinity of cyt c vs. R5P-glycated cyt c (1 mM; 3 d 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 as 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 to reduce 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 vs. 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 R5P modified. Thus, the relative amount of signal from fragments containing the altered Lys/Arg and the fragment immediately behind this site will reduce in intensity upon increased modification. At 24 h of reaction, only Lys8 has significant levels of modification at approximately 35% (see Supplemental 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 loss or gain in intensity upon R5P glycation for three days at 37 °C (n=6). At six days of reaction, the results indicate that Lys8, Lys72, Lys87 or 88, and possibly Lys25 are the four sites modified by levels greater than 70%. Several other sites have moderate modification amounts. (See Supplemental 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 mM 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 zero hour incubation activity. (This was done since different activities of COX were used in some of the experiments.) Incubated for a 24 hours at 37 °C, the un-treated cyt c retained approximately 80% of its original rate capacity of transfer of electrons to COX while the 10 mM and 50 mM R5P-treated cyt c showed significantly ($p < 0.05$) lower ability

to transfer electrons at only 55 % and 12 %, respectively. (At 6–8 h of incubation, the 50 mM R5P-treated protein showed a significant difference to the control while the 10 mM R5P-treated cyt c did not.) A similar trial using glucose 6-phosphate over a three day period did not show any significant difference to 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 uni-lamellar liposomes for 1 – 4 h at 37 °C and then micro-filtered through a 100,000 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 higher association occurring at the lower ionic strengths provided by the 10 mM HEPES buffer (29). Table 1 illustrates the 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 2.5 h R5P-treated cyt c vs. untreated cyt c while a decreased binding (approximately 50 %) was observed for the treated cyt c when employing a longer R5P glycation time of 18 h. We obtained similar results for experiments using large, multi-lamellar liposomes (Table 1) except significant differences in binding were observed at the 2.5 h incubation samples and overall the larger liposomes had a greater binding nature. The differences in the association of glycated vs. unglycated cyt c to cardiolipin-containing liposomes were also seen at other liposome:cyt c ratios (1:2 to 1:20) with an expected higher 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 $4 \times 10^5\ M^{-1}$ is obtained. The K_B for cyt c held for 18 h was less, presumably because of some cyt c denaturation. Comparatively, the binding constant for cyt c is approximately eightfold that of the R5P-glycated cyt c. Although the binding relationship of cyt c to liposome components has yet to be clearly defined, it is worth noting that the number of cyt c 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 equimolar ATP caused the affinity of the resultant cyt c for uni-lamellar liposomes to be increased (Table 1). In experiments using multi-lamellar 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 greater tendency to bind to liposomes than bovine cyt c. (In order 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 decreased the ability of the protein to bind to the uni-lamellar liposomes (Table 1). In the same manner as bovine cyt c, the presence of ATP during the R5P-cyt c incubation increased 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 non-glycated protein. When the relative amount of mitoplast was varied in the incubation mixture, the non-glycated 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 mitoplast amount. 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 cyt c association 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; 37°C) using multi-lamellar 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 1:5 with uni-lamellar 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 18 h of reaction, 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 multi-lamellar liposomes was faster despite a lower 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 (multi-lamellar) (Table 1 and Fig. 4). ATP placed directly in the reaction mixture of untreated ferro-cyt c and liposomes caused a slowing of the spectral change rate (2-fold change in $t_{1/2}$ using multi-lamellar liposomes) that was equal to that caused by simply raising the ionic strength using NaCl. Thus, the role of ATP to limit the effect of R5P glycation on cyt c’s liposome binding is in opposition to the effect normally seen by ATP on untreated cyt c’s association to membranes.

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

The spectral change profiles did not appear to be consistent with the data we were receiving 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 for the percent bound (Fig. 5). However, at the end of one hour incubation at 37 °C with multi-lamellar liposomes – the point where we saw complete spectral change indicating a full shift to the high spin state 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 form.

The A_{550} and A_{526} absorbance curves themselves varied between trials when using multi-lamellar 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 time with the liposomes (see Fig. 4), the samples untreated with R5P gave A_{526} curves (inset, Fig. 4) that remained of constant value 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 glycated cyt c interaction with liposome showed an A_{526} curve that simply held constant in absorbance (see inset, Fig. 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 (Fig. 6) showing the multi-lamellar liposomes themselves (without reaction with cyt c) to be relatively small (up to 15 μm, Fig. 6A); the liposomes reacted with glycated cyt c to be somewhat larger (up to 30 μm, Fig. 6C) but still spherical; and the liposomes reacted with un-treated cyt c to form large lipid aggregates (of up to approximately 100 μm, Fig. 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 turbidity increase 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 approximately twofold larger in average size than those exposed to glycated cyt c, which were on average threefold larger than unexposed mitoplasts (see Supplemental Information).

A similar set of experiments (microfiltration and spectral change to liposomes) were performed using D-glucose as the glycating sugar. At one week of reaction at 37 °C, glucose-glycated cyt c incubated with multi-lamellar liposomes (1:5) showed little change in binding vs. 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 one week at 37 °C was relatively insignificant.

A third technique for assessing cyt c association 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 towards the cathode unless it is bound to the negatively charged liposomes. When the ratio of liposome to cyt c increases, more cyt c is attached and the membrane-bound cyt c is directed towards the anode (Fig. 7). Untreated cyt c has been shown to shift its movement towards 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 (Fig. 7, Lane 5). As mentioned previously, glycation of cyt c by R5P makes the protein more negatively charged, and it migrates more rapidly towards the anode (Fig. 7, lane 7 vs. lane 8). Except for a small population of cyt c, treatment of glycated cyt c (18 h, 37 °C) with uni-lamellar liposomes did not significantly change the protein's electrophoretic mobility (Fig. 7, lanes 1 – 3 vs. lane 7) while a significant change in mobility was observed for untreated cyt c (Fig. 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 d; 37 °C) ferro-cyt c did not change significantly (initial rates at 416 nm: ~0.175 A/min) from the untreated sample. At 6 d of 37 °C incubation, 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 twofold in both cases) for these samples after they had been reacted with liposomes.

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 (Lys 8,13,27,72,87) (3) which 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 non-polar 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 acyl chain insertion at one or two cavities within the cyt c interior (12, 14, 29). Additionally, cyt c also houses an ATP binding site which includes a group of lysines (Lys72,86,87) and a critical arginine, Arg91 (4, 33). ATP binding to this site has been speculated 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 reducing 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 R5P association with and glycation of cyt c affects 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 at this site or at least involves some of the ATP-interacting residues including Lys86, Lys87, Lys88, Arg91 or perhaps Lys72 and Lys73. 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. In 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 implicate 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 vs. 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 pKa's of the various lysines are likely not significantly different to 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, are 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 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 which involves Asn-52 (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 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 acyl chain penetration into the cyt c protein. Their results suggest that electrostatic interactions not only are important for initial association but also for maintaining the acyl chain insertion. In other words, the acyl chain insertion is not necessarily a permanent state. They also observed binding and the low spin 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 of 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 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 heme protein's association to 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 liposome (microfiltration studies) at 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 which have acyl chain insertion. However, our microfiltration binding studies clearly show a difference in cyt c-multi-lamellar liposome binding between the 1:8 and 1:20 at 1 h of reaction while the spectral change results indicate that all of the cyt c molecules have shifted from the low spin to the high spin, 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 where the initial association of cyt c onto the cardiolipin-containing membrane results in a conformation change (to the β-sheet form) and then the associated cyt c would undergo the acyl chain insertion and permanent binding at either site A or site C 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) which showed greater binding affinity for the mammalian cyt c.] Similar to the bovine form, glycation of yeast cyt c increased $t_{1/2}$ of the spectral change and decreased the binding affinity of the protein to associate with membranes. The ability of ATP to reverse the R5P effects on yeast cyt c are 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 reacted with cardiolipin-containing multi-lamellar liposomes.

Glycation affects membrane association. The spectral studies and the binding studies are in agreement in showing that a 2.5 hour and longer glycation with R5P inhibit the 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 it may cause steric difficulties for the cyt c to closely interact 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 turbidity change for the R5P-glycated cyt c reaction with multi-lamellar 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 one 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 which directs it to a highly positively 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 in pro-apoptotic events. Along with its intriguing ability to rapidly reduce cyt c, the sugar phosphate attaches readily onto 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 cyt c's affinity to 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*.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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ABBREVIATIONS

ABTS	2,2′-Azino-bis(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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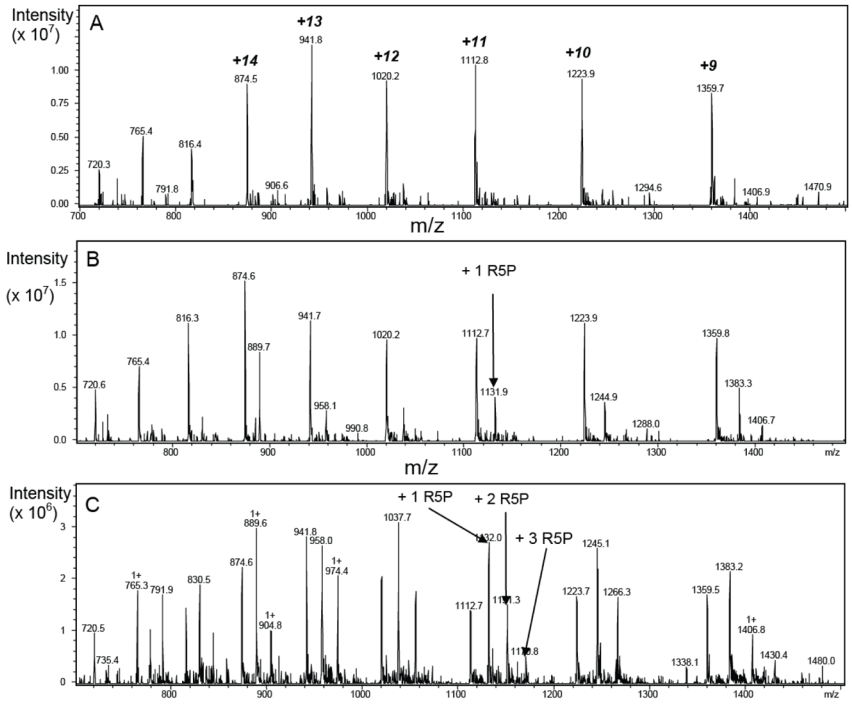

Figure 1.

R5P reaction with bovine heart cyt c results in attachment of 2 – 3 R5P molecules to the protein over an 8 h period. Each figure 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. Incubation time of the cyt c with R5P (10 mM) at 37 °C were 0 h (A), 2 h (B), and 8 h (C). The development of new spectrum 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 correspond to the addition of +212, +424, and +636 daltons which clearly represent the addition of 1, 2, and 3 R5P molecules.

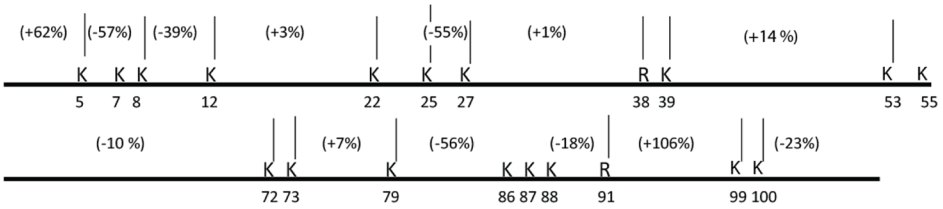

Figure 2.

A 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.

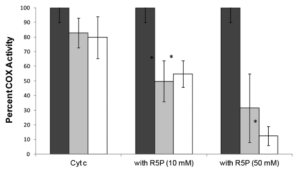

Figure 3.

The 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 activity of the 0 h activity. The black bars are 0 h incubation of cyt c with R5P; the gray bars are 6 – 8 h incubations, and the white bars are 20 – 24 h incubation. The error bars are ±1 standard deviation. Data marked by an asterisk (*) indicates significance ($p<0.5$) vs. the untreated cyt c control.

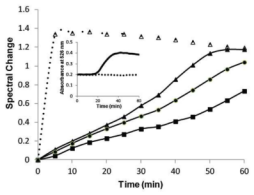

Figure 4.

R5P glycation slows the spectral change of cyt c upon its incubation with cardiolipin-containing multi-lamellar liposomes. The rate of spectral change ($\Delta;A_{550} - A_{526}$; see text) is shown upon the reaction of cyt c (0.5 mg/mL) with liposomes (molar ratio = 1:5) at 37 °C for one hour.Pretreatment of cyt c (37 °C; 24 h): Untreated bovine cyt c (—▲—); bovine cyt c incubated with R5P (10 mM) ■—); 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, 24 h) (·····Δ·····) is shown. Inset: The A_{526} curves for untreated bovine cyt c (——) and R5P glycated bovine cyt c (·····) mixed with multi-lamellar liposomes show turbidity changes for the untreated cyt c that are not observed for the glycated protein.

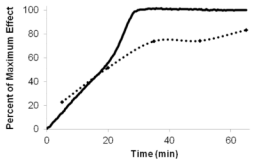

Figure 5.

Spectral change correlates with binding (microfiltration analysis) with incubation time of interaction of bovine cyt c (0.5 mg/mL) with multi-lamellar liposomes (1:5 molar ratio) but does not agree with the extent of binding at longer incubation times. Both graphs are showing "association" on a percentage scale of maximum observed effect. The spectral change (——) and the binding percentage (·····◆·····) both increase from 0 % to 60 % with the same time scale but while the spectral change increases to 100 %, the microfiltration levels at this cyt c:liposome ratio at only 80 %.

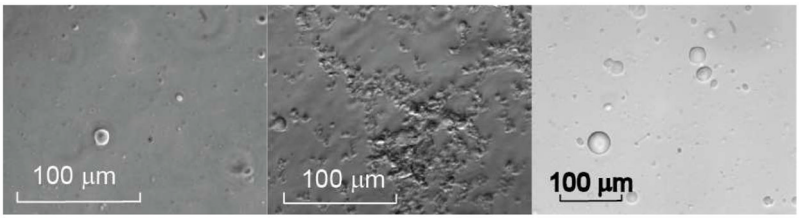

Figure 6.

Microscopic analysis of cytochrome-liposome interaction shows that R5P glycation of cyt c reduces the protein's tendency to form large aggregates. Panel A: Multi-lamellar liposomes untreated with cyt c. An average size of the liposomes were approximately 10 μm. Panel B: Multi-lamellar liposomes treated with untreated cyt c for one hour at 37 °C. The aggregate in the center of the picture is approximately 100 μm in diameter. Panel C: Multi-lamellar liposomes treated with R5P-glycated cyt c for one hour at 37 °C. The typical size of the individual liposomes is approximately 35 μm in diameter.

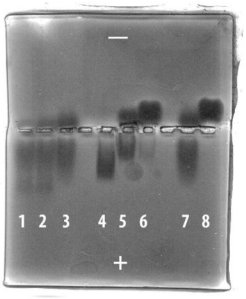

Figure 7.

R5P glycation reduces the association of bovine cyt c for cardiolipin-containing liposomes. Agarose gel electrophoresis of R5P-treated or untreated cyt c incubated with uni-lamellar 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 held for 18 h at 37 °C. Samples were reduced and microfiltered prior to electrophoresis.

Table 1

Spectral Change and Liposome Binding Results

Incubation Time		Binding Agent (ratio)‡	Spectral Change $t_{1/2}$ (min)	Binding Free Cyt c (%)
	Bovine Cytochrome c (1 mg/mL)			
2.5 h	untreated	Liposomes (1:5)	92 ± 5	6.8 ± 0.5
	with R5P (10 mM)	(uni-lamellar)	124 ± 8	7.1 ± 0.3
18 h	untreated	Liposomes (1:5)	86 ± 6	44 ± 4
	with R5P (10 mM)	(uni-lamellar)	145 ± 2	73 ± 4
	with R5P (10 mM) + ATP (10 mM)		101 ± 4	51 ± 2
24 h	untreated	Liposomes (1:5)	23 ± 6	23 ± 13
	with R5P (10 mM)	(multi-lamellar)	47 ± 6	71 ± 21
	with R5P (10 mM) + ATP (10 mM)		21 ± 1	30 ± 1
24 h	untreated	Mitoblasts (1.4:1)	--	12 ± 5
	with R5P (10 mM)		--	63 ± 6
	Yeast Cytochrome c (1 mg/mL)			
18 h	untreated	Liposomes (1:1)	12	37
	with R5P (10 mM)	(uni-lamellar)	54	49
	with R5P (10 mM) + ATP (10 mM)		8	37

All error ranges given are ± 1 standard deviation.

‡For liposomes, molar ratios of cytochrome c to total lipid are given. For mitoblasts, ratios are protein ratios of cytochrome c to mitochondria. Binding values are for 1–4 h incubation at 37 °C.