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PROBING ELECTRON TRANSFER AND THERMAL STABILITY OF GLYCATED BY LACTOSE CYTOCHROME C

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ლაქტოზით გლიკირებული ციტოქრომ C-ს ელექტრონული მიმოცვლის და თერმული სტაბილურობის კვლევა

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რეზიუმე

შესწავლილია ლაქტოზით გლიკირებული ციტოქრომ C-ს ელექტრონული მიმოცვლის სიჩქარე და თერმული მდგრადობა გლიკირების 14 დღიან პერიოდში (T = 25 ^{0}C). ციტოქრომ C-ს და მოდიფიკატორით დაფარულ ოქროს ელექტროდს შორის ჟანგვა-აღდგენითი რეაქციის სიჩქარე და თერმოდინამიკური მდგრადობა ცილის გლიკირების პროცესის სხვადასხვა ეტაპზე გაზომილი იქნა, შესაბამისად, ციკლური ვოლტამპერომეტრიის და მიკრო კალორიმეტრული მეთოდის გამოყენებით. მიღებული ექსპერიმენტული შედეგები მიუთითებს იმაზე, რომ ლაქტოზით გლიკირება იწვევს ციტოქრომ C-ს რედოქს აქტივობის მკვეთრ გაუარესებას გლიკირების პერიოდის დაწყებიდან ერთი კვირის შემდგომ, მაშინ როცა ცილის გლობალური თერმული სტაბილობა შენარჩუნებულია გლიკირების მინიმუმ ორკვირიან პერიოდში.

Introduction. In our previous work [1], we studied the effect of the prolonged glycation process by monosaccharide-D-Glucose on the electron transfer properties of Cytochrome C (Cyt C). Cyt C is a small (ca 12 KDa), water-soluble, heme-containing protein, which performs the function of electron carrier in the mitochondrial respiratory chain (shuttling electrons between enzymes Cyt C reductase and Cyt C oxidase), as well as plays a key role in apoptosis-programmed cell death process [1-5]. Based on the analysis of the data of voltamperometric measurements gained during the long-term glycation process of Cyt C (approximately three-week incubating period in 1M Glucose solution, T= 25^{0}C), authors [1] concluded that glycation by glucose does not affect protein's electron transfer properties significantly. Cyt C preserves its redox activity for three weeks [1]. At the same time, according to our data, prolonged glycation of Cyt C with glucose leads to a significant increase in the protein's thermal stability (unpublished data).

Different glycation agents can affect the structure and electron transfer properties of proteins in general and in the case of Cyt C specifically [6-10]. According to [6-8], glycation of Cyt C with glucose, glycosyl, and methylglyoxal is a slow process (taking several weeks to affect the protein's native structure significantly). In contrast, the glycation process by sugar ribose 5-phosphate takes place much more (approximately 100 times) faster and induces deterioration of the protein's electron transfer properties [8]. The effect of polysaccharide–lactose on the Cyt C was studied in [9-10]. Data from circular dichroism spectral studies [9] shows that even a high glycosylation level of Cyt C by lactose causes some minor structure and activity changes, increasing the stability of Cyt C and does not affect the protein's apoptosis capability [9]. According to [10], natural and chemical glycosylation of Cyt C by lactose increases proteins thermodynamic stability, like the case of glucose as a glycan [11]. To our knowledge, no data on the kinetics of the lactose-glycated Cyt C can be found in the literature.

In the present work, we investigate the global stability and electron transfer characteristics of Cyt C under a prolonged glycation process by lactose. Lactose is characterized by relatively low solubility in water solutions -18,9 g/in 100g solution (0.55 M) at 25^{0}C [14]. So, experiments were performed at 25 ^{0} under the maximum concentration of lactose that dissolves at a given temperature.

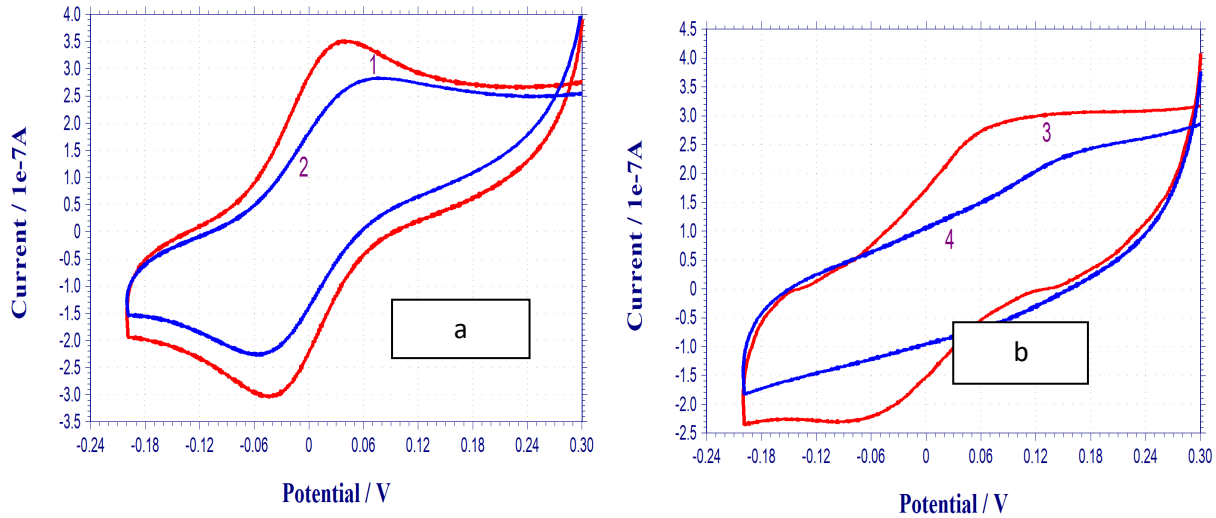
Differential scanning calorimetric measurements were performed for assessment of the conformational degree of glycated Cyt C. Direct kinetic studies of electron transfer properties of Cyt C in lactose-containing solutions were performed using cyclic voltametric method [13], where the modified gold electrode was used as electron acceptor. The use of a modifier (4,4'-bipyridyl) is due to the fact, that the protein adsorbed onto a bare metal electrode surface denature (caused by the strong electrostatic interaction with the surface) and loses its activity [11,12]. So, as previously [1,12], we covered the gold electrode with a modifier 4,4'-bipyridyl, which forms an adsorbed layer on the electrode surface and facilitates the electron-transfer reaction.

Materials and Methods. Horse heart Cyt C, NaCl, Na_2HPO_4, 4,4'-bipyridyl (BP), and Lactose were purchased from Aldrich and used as received. All solutions were prepared using MilliQ water. Kinetic experiments were performed with a conventional three-electrode system: 2 mm Ø gold disc sealed in Teflon cylinders (BAS) was used as the working electrode, platinum wire as the counter electrode, and Ag/AgCl/3M NaCl as reference electrode. Before each experiment, the working electrode was sequentially polished with Alumina water slurry on a Buehler polishing pad, rinsed with water, and modified by immediately dipping the gold electrode in 0,01 M solution of 4,4'-bipyridyl for 30 min. Voltamperometric measurements were carried out with Potentiostat CH Instrument Model 1200B controlled by an external PC under Windows 98/NT/Me/2000/XP/Vista/7/8. Microcalorimetry measurements were performed with the DSC instrument DASM-4. All experiments were performed at T= 25^{0}C.

Results and Discussions. Figure 1 (a,b) shows cyclic voltametric (CV) curves of Cyt C electron transfer response recorded at BP-modified gold electrode in potassium chloride buffered solution in the presence of 0,55 M lactose at different periods of Cyt C glycation process.

As shown in Fig. 1a (curve 1), the reduction and oxidation response of Cyt C recorded up to 7 days of the glycation process is well defined. Deterioration of CV curves shape starts on the 8-th day of the glycation process: voltamprometric response gradually becomes less defined (Fig.1 a,b; curves 2,3), peak height decreases, and finally, on 13-th day of the glycation process, CV peaks disappear Fig.1b (curve 4).

Fig. 1. Typical CV curves of the electron transfer process for Cyt C in 0.15 M NaCl + 20 mM PBS + 4 mg/mL Cyt C + 0.55M M lactose solution at different stages of glycation process: a) 1-day 1 to 7; 2-day 8; b) 3-day 12; 4- day 13. v=0.02 V/sec, T= 25 ^{0}C.



Values of peak-to-peak separation(ΔEp), calculated from experimental current-voltage curves (Fig.1) for different periods of the glycation process, are presented in Table 1. The value of diffusion coefficient (D = 1,1 x10 $^{-7}$ cm ^{2}/s) of Cyt C was calculated from the values of reductive peak current, Ip, (Fig.1, curve 1) by using eq (1) [12,13]

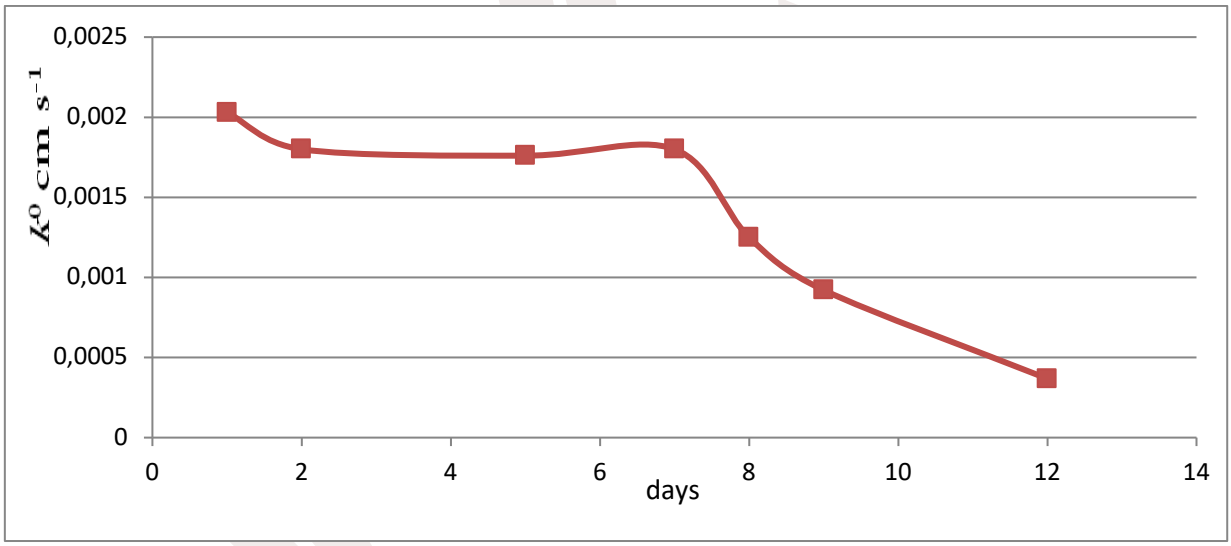
$$Ip = -0.446nF(nF/RT)^{1/2}CoD^{1/2}\upsilon^{1/2} \quad (1)$$

where n is the number of transferred electrons (here n =1), F is the Faraday constant, Co is the reactant Cyt C concentration in the bulk, R- gas constant, T- temperature, v-scan rate. Using experimental CV peak-to-peak separation and experimental diffusion coefficient values, the heterogeneous standard rate constant(k^0) of the electron transfer reaction of Cyt C was calculated according to Nickolson's method [1,13]. Data are presented in Table 1 and Fig. 2.

Table 1. Values of kinetic parameters for Cyt C in 0.1 M NaCl + 20 mM PBS + 4 mg/mL Cyt C + 0.55 M lactose solution at different stages of the glycation process.

Time (days)	Day 1- day7	Day 8	Day 9	Day 12	Day 13
ΔEp, mV	83 ± 2	121	137	≈ 262	Nonmeasurable
K^0 cm s^{-1}	1.9 ±0.1x10^{-3}	1.25 x10^{-3}	9.21 x10^{-4}	3.68 x10^{-4}	Nonmeasurable

Fig. 2. Values of rate constant of Cyt C at different incubation periods in 0.1 M NaCl + 20 mM PBS + 4 mg/mL Cyt C + 0.55 M lactose solution.



Experimental data displayed in Table 1 and Fig. 2 shows that the electron exchange properties of a protein are slightly affected by the lactose during a glycation period of up to 7 days. Starting from day 8, during the further 5 days of the glycation process (day 8 to 12), value of peak-to-peak separation increases, which is reflected in the sharp decrease of the rate constant to a value beyond which it cannot be measured by the given method (starting from the 13-th day of the glycation process).

Fig.3 shows zero-baseline-corrected calorimetric protein melting curves (partial heat capacity of protein versus temperature) for glycated by the lactose Cyt C at different periods of the glycation process.

Values of transition temperature (Tm), overall melting enthalpy ($\Delta Hcal$,) and peak width at the half height (ΔT) for glycated by lactose Cyt C at different stages of the glycation process calculated from the curves depicted in Fig. 3 are gathered in Table 2.

Fig 3. The DSC melting curves of glycated by lactose Cyt C in 0.15 M NaCl + 20 mM PBS + 2 mg/mL Cyt C + 0.55 M lactose solution at different stages of the glycation process. Left-Day 1; Right- day 14.

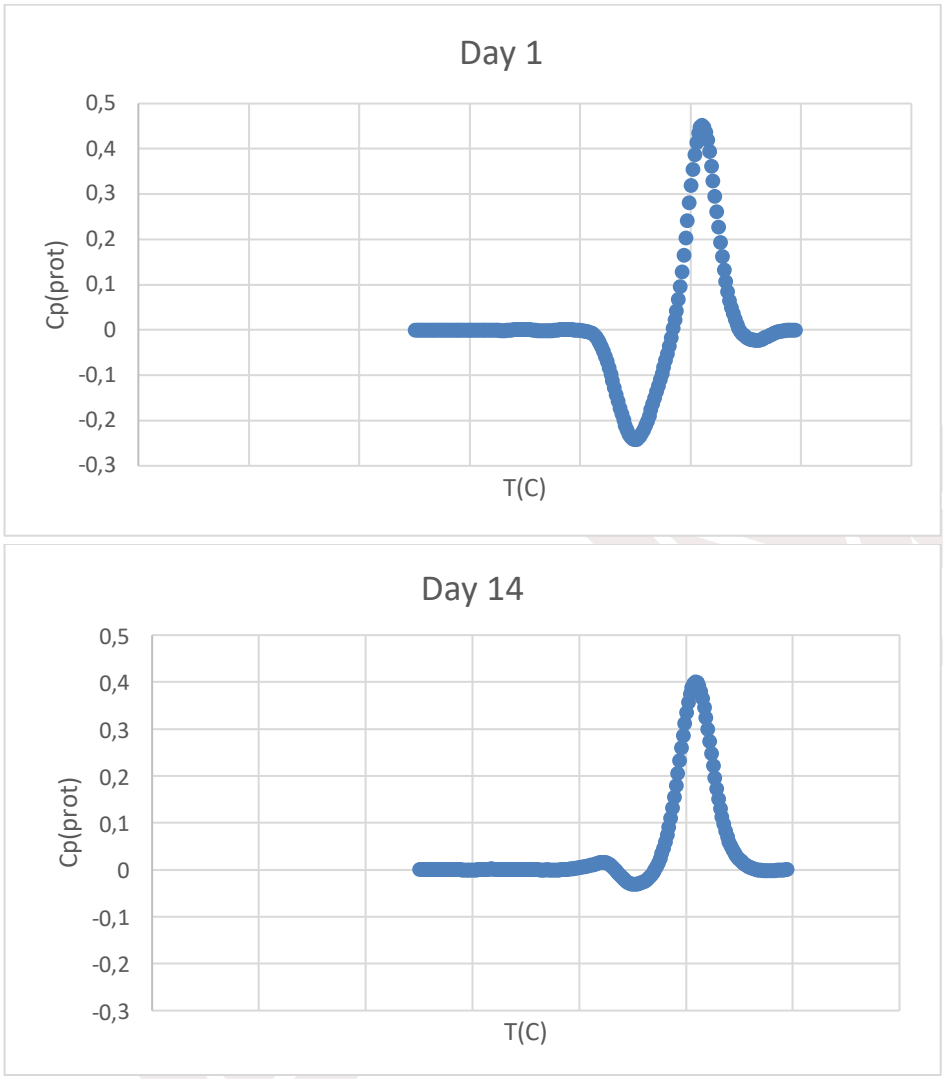


Table 2. Values thermodynamic parameters for Cyt C in 0.1 M NaCl + 20 mM PBS + 4 mg/mL Cyt C + 0.55 M lactose solution at different stages of the glycation process.

Time (days)	Tm, [ºC]	ΔT, Δ ΔTm, [ºC]	ΔHcal, (arbitrary units)
Day 1	101,8	7.75	7.08
Day 14	102.1	7.93	5.18

It is visible from Fig. 3 that, the melting (endothermic) curve for Cyt C maintains its peak shape during a glycation period of up to 14 days. The data presented in Table 2 shows, that the transition temperature and other thermodynamic parameters also do not change significantly, indicating that the global structure of Cyt C is not considerably affected by lactose during the observed two-week period. We do not discuss the small negative pre-peaks visible on the thermograms (Fig.3), likely due to protein aggregation effects.

The combined analysis of the obtained voltamperometric and thermodynamic results for Cyt C shows that, unlike glucose [1], glycation with lactose has a different effect on the stability and kinetics of Cyt C. In particular, the obtained thermodynamic parameters indicate that the tertiary structure of Cyt C

is stabilized in the presence of lactose in the solution (value of transition temperature of non-glycated Cyt C Tm= 75,5^{0}C [11]) and maintains its stability at least during the 14-day glycation period. At the same time, the kinetic characteristics of lactose-glycated Cyt C deteriorate dramatically after a week of the glycation process, making it impossible to detect the electron exchange signal of the protein after the twelfth day of the glycation period.

Thus, it can be said that during the long-term (up to two weeks) glycation period of Cyt C with lactose, the global stability of the protein is maintained while heme activity is degraded. The obtained result confirms the opinion expressed in the literature that local and global protein stability do not automatically follow each other. Indeed, this work [11] shows different trends in global thermodynamics and the local heme (Met80-Fe^{3+}bond) stability of Cyt C in the presence of destabilizing agent urea.

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PROBING ELECTRON TRANSFER AND THERMAL STABILITY OF GLYCATED BY LACTOSE CYTOCHROME C

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SUMMARY

The electron exchange process and thermal stability of the lactose-glycated Cytochrome C (Cyt C) during the 14-day glycation period (T = 25 ^{0}C) were observed. The oxidation and reduction reaction rate of Cyt C at a modifier-coated gold electrode, as well as the thermodynamic stability of protein at different stages of the glycation process, were measured using, respectively, cyclic voltammetry and microcalorimetric methods. The obtained experimental results indicate that glycation with lactose leads to a sharp deterioration of the redox activity of Cyt C one week after the beginning of the glycation period. At the same time, the global thermal stability of the protein is maintained for at least two weeks of the glycation period.

Keywords: Cytochrome C, Glycation, Lactoze, cyclic voltammetry, calorimetry, electron transfer.