Novel approach to protein-protein interaction assessment

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Abstract. Past decades, the study of proteins and small molecules interaction has been at least lagging behind the study of other types of interactions such as protein-protein, protein-DNA and protein-RNA. The aim of this study is to evaluate the effect of low-molecular biologically active compounds, lactate and pyruvate, on protein-protein interactions and to provide quantitative assessment. Using AB0 blood groups system model and labeled monoclonal anti-A and anti-B antibodies we studied the effect of natural metabolites lactate and pyruvate on antigen-antibody interaction. Erythrocytes of A(II) and B(III) blood groups were incubated with lactate and pyruvate before the agglutination reaction. Then the agglutination reaction was performed. Visualization of agglutination complexes was carried out by means of confocal laser scanning microscopy with further processing of noise pixels. A series of in vitro experiments with further visualization and mathematical processing showed the co-directed but more pronounced effect of lactate on the antigen-antibody reaction as compared to pyruvate. In case of influence on antigen A the aggregation process intensification was noted, while this process was inhibited in case of influence on antigen B. The obtained results demonstrate possibility of using small molecules, in particular lactate and pyruvate, as molecular probes and prospects of erythrocytes with antigenic determinants of AB0 system expressed on their membranes for studying protein-protein interactions due to visualization clarity and possibility of quantitative assessment of this process. It is proved that lactate and pyruvate can act as regulators of protein-protein interactions on the example of antigen-antibody reaction.

1. Introduction

With the development of such sciences as genomics, proteomics, and metabolomics, more and more data on intermolecular interactions, in particular protein-protein interactions, are accumulated [1]. They play the most important role in realization of vital functions in cells, organs and in organism in general. However, protein-ligand interactions are regulating the direction and setting the pace of intra- and extracellular processes [2,3]. The knowledge about the low molecular ligands with which proteins can interact is insufficient [4].

In the center of our interest are the key intermediates with a similar chemical structure - lactate and pyruvate, which are the redox pair and play an important role in the cell energy metabolism. Lactate was previously considered a dead end of anaerobic metabolism. Today it is known that lactate formation occurs continuously not only in anaerobic but also in aerobic conditions [5]. According to the literature, lactate is the main source of energy, a signal molecule, and hormone-like compound [6,7]. Pyruvate is a metabolite that links the cytosolic and mitochondrial metabolic pathways and there is evidence of protective, antioxidant and anti-inflammatory effects [8,9,10]. At the same time, the area of influence of these metabolites on protein-protein interaction processes remains insufficiently studied.
It is important for a researcher to not only establish the fact of interaction of a low-molecular ligand with the protein molecule of interest, but also to be able to quantitatively assess the changes caused. From this point of view, it is promising to study the protein-protein interaction using the model system of AB0 blood groups, represented by antigenic glycoproteins A and B, natural and monoclonal antibodies [11, 12]. However, previously described approaches allowed only qualitative assessment of the observed changes. Next step was to move from qualitative assessment to quantitative one.

2. Aim of the study
The aim of the study was to evaluate the effect of low-molecular biologically active compounds, lactate and pyruvate, on protein-protein interactions and to provide quantitative characteristics.

3. Materials and methods
The object of our study are erythrocytes obtained by venipuncture. Red blood cell count was carried out on the hematological analyzer Sysmex KX 21 (Roche Diagnostics, Switzerland), then the dilution of 1x10⁶/1 erythrocytes was achieved using the FAX flow saline solution. Pre-incubation of 100 µl of the obtained erythrocyte dilution with 20 µl lactate or pyruvate in the final concentration of 2 mM was performed for 5 minutes. Subsequently, the agglutination reaction of glycoproteins A and B with specific labeled antibodies was carried out: monoclonal conjugated antibodies Blood group A antigen (Z2A), Blood group B antigen (89-F). Fluorescein isothiocyanate (FITZ) from Santa Cruz biotechnology, Inc. (USA) was used as a fluorescent label. The control sample was free of lactate and pyruvate. The reaction time was 20 minutes in a darkened room.

Visualization of protein-protein interaction was performed using an experimental unit created on the basis of confocal optical microscope (Olympus, Japan) and laser combine by ANDOR. Microphotographs were registered in two modes: basic scattering and fluorescence. In the scattering mode the source of radiation was a halogen lamp, in the fluorescence mode - 100 mW radiators, wavelengths 488 and 561 nm.

Processing of the obtained microscopic images was carried out in JMicroVision 1.2.7 and Andor IQ software. To reduce noise and increase contrast of microscopic images a filter with a threshold of about 5% of the maximum frame intensity was used and it was replaced by a zero signal. The size of the received micro photos was 400x400 µm, resolution - 400 nm per pixel. The agglutination conglomerates were assessed (the criterion was the presence of more than 2 erythrocytes in the complex) on the main scattering microscopic images, the area of complexes in square micrometers and the percentage occupied by one complex in the frame were calculated. The whole frame with fluorescent complexes was isolated on fluorescent microphotographs and the mean green pixels’ intensity in the frame was calculated.

Statistical processing of the obtained research results was carried out in the SPSS 21 (IBM SPSS Statistics, USA, license # 20130626-3). The calculated features had a normal distribution, proved by Kolmogorov-Smirnov criterion. For this reason, we used parametric methods of statistical analysis. We found mean (M), error of mean (m), 95% confidence interval (95% CI). Intergroup comparisons were made using the Student criterion for two independent samples.

4. Results and discussion
To reveal and quantitatively assess influence of the studied metabolites on protein-protein interaction we used the AB0 blood groups model system, where the protein-protein interaction was observed between membrane glycoproteins of erythrocytes A and B and monoclonal antibodies with fluorescent label.

Figure 1 shows microphotographs of the antigen-antibody interaction of glycoprotein A with anti-A monoclonal antibodies in the basic scattering and fluorescence mode. The mathematical processing of the results obtained in the basic scattering mode is shown in table 1.
Figure 1. Microphotographs of antigen-antibody complexes. a-c - basic scattering mode: a - glycoprotein A (control), b - glycoprotein A + lactate, c - glycoprotein A + pyruvate; d-f - fluorescence mode: d - glycoprotein A (control), e - glycoprotein A + lactate, f - glycoprotein A + pyruvate.

Table 1. Effect of lactate and pyruvate on glycoprotein A (basic scattering mode).

|                          | Control          | glycoprotein A 2mM | glycoprotein A 2mM |
|--------------------------|------------------|---------------------|---------------------|
| Number of complexes      | 16.25 ± 0.48     | 48.75 ± 7.36        | 20.5 ± 5.9          |
| M ± m                    | 14.73 – 17.77    | 25.31 – 72.19       | 1.74 – 39.26        |
| 95% CI                   | 0.004            | 0.5                 |                     |
| p                        |                  |                     |                     |
| Mean area of one complex, μm² | 499.2 ± 64      | 759.65 ± 128.7      | 518.6 ± 149.2       |
| M ± m                    | 371.36 - 627     | 505.75 - 1013.55    | 221.67 - 815.53     |
| 95% CI                   | 0.04             | 0.5                 |                     |
| p                        |                  |                     |                     |
| Mean area occupied by one complex in the frame, % | 0.31 ± 0.04 | 0.48 ± 0.08 | 0.32 ± 0.09 |
| M ± m                    | 0.23 - 0.39      | 0.32 - 0.63         | 0.14 - 0.51         |
| 95% CI                   |                  |                     |                     |
| p                        | 0.04             | 0.5                 |                     |
| Mean sum of areas occupied by all complexes in the frame, % | 5.04 ± 0.62 | 23.4 ± 1.95 | 6.56 ± 3 |
| M ± m                    | 3.06 - 7         | 16.70 - 29.13       | 2.95 - 16.09        |
| 95% CI                   | 0.0001           | 0.63                |                     |
| p                        |                  |                     |                     |

It was found that pre-incubation of glycoprotein A with lactate leads to a significant increase in the number of antigen-antibody complexes - 48.75 ± 7.36 (p=0.004); the mean area of one complex - 759.65 ± 128.7 μm² (p=0.04); the mean area occupied by one complex in the frame 0.48 ± 0.08 % (p=0.04) and the mean sum of areas occupied by all complexes in relation to the whole frame 23.4 ± 1.95 % (p=0.0001) in comparison with the control. At the same time, adding pyruvate to the system causes similar changes, but no significant differences from the control sample were revealed. The effects of
lactate and pyruvate on the antigenic determinant A are co-directed and stimulating, but differ in the response degree: lactate addition leads to the formation of more complexes in comparison with pyruvate (p=0.02), which occupy a larger mean area (p=0.003).

Microphotographs of glycoprotein B interaction with the investigated metabolites are presented in figure 2.

According to mathematical processing of microphotographs (table 2), pre-incubation of antigen B with lactate and pyruvate causes inhibition of antigen-antibody reaction that is manifested in reduction of formed agglutination complexes number in comparison with control: lactate - 17 ± 0.6 (p<0.0001) and pyruvate – 32.75± 1.03 (p<0.0001), decrease in mean area of one complex at interaction with lactate 390 ± 87 μm² (p=0.0006), pyruvate - 302.5 ± 25.27 μm² (p=0.0006) and decrease in the mean area occupied by one complex in the frame of pre-incubation with lactate 0.24 ± 0.054 % (p=0.001) and pyruvate 0.19 ± 0.016 (p<0.0001). It should be noted that in this case the effect of metabolites on the experimental system was co-directed and inhibiting, but the difference of lactate and pyruvate effect was observed only in the number of formed complexes (p=0.0001), for other parameters no difference between the effect of pyruvate and lactate was found.

**Table 2.** Effect of lactate and pyruvate on glycoprotein B (basic scattering mode).

|                      | Control                  | glycoprotein B            |
|----------------------|--------------------------|---------------------------|
|                      | 55.5 ± 1.7               | 17 ± 0.6                  |
| Number of complexes  | 95% CI 50.22 - 60.78     | 29.47 - 36.03             |
|                      | p< 0.0001                | p< 0.0001                 |
|                      |                          | p lactate-pyruvate = 0.0001|
| Mean area of one complex, μm² | 697.4 ± 39.5 | 390 ± 87                  |
|                      | 95% CI 619.62 - 775.26   | 252.48 - 352.46           |
|                      | p<0.0001                 | p<0.0001                  |
|                      | p lactate-pyruvate = 0.197|
| Mean area occupied by one complex in the frame, % | 0.44 ± 0.025 | 0.24 ± 0.054 |
|                      | 95% CI 0.44 - 0.24       | 0.19 ± 0.016              |
|                      | p<0.0001                 | p<0.0001                  |
The presented analysis of microphotographs of antigen-antibody reaction in the basic scattering mode is quite accurate, however, does not allow to exclude a number of non-specific interactions, which may contribute to the visible aggregation effect. To improve the accuracy of interpretation, table 3 presents the results of mathematical processing of microphotographs in the fluorescence mode, in which the emission is registered only from antigen complexes and its corresponding monoclonal antibody labeled with fluorophore.

**Table 3. Effect of lactate and pyruvate on glycoproteins A and B (fluorescence mode).**

| Glycoprotein A | Glycoprotein B |
|----------------|----------------|
| **Mean green pixel intensity, c.u.** | **Control** | **Lactate 2mM** | **Pyruvate 2mM** | **Control** | **Lactate 2mM** | **Pyruvate 2mM** |
| **M ± m** | 6.2 ± 0.98 | 13.4 ± 1.97 | 6.8 ± 0.1 | 2.7 ± 0.05 | 0.2 ± 0.04 | 0.28 ± 0.073 |
| **95% CI** | 3.45 - 8.90 | 7.09 - 19.64 | 6.41 - 7.11 | 2.51 - 2.85 | 0.05 - 0.32 | 0.05 - 0.52 |
| **Change, %** | +116.5 ± 0.01 | +9.5 | +0.62 | -93 | <0.0001 | <0.0001 |
| **p** lactate-pyruvate= | 0.01 | | | | | |
| **p lactate-pyruvate=** | | | | | | 0.3 |

The results obtained follow the trend in tables 2 and 3 above. The addition of lactate and pyruvate into the experimental system with antigen A leads to an increase in the mean green pixel intensity in the frame, while the lactate effect is more pronounced than that of pyruvate and is 13.4 ± 1.97 c.u. compared to 6.8 ± 0.1 c.u., the difference in the metabolite effect is significant (p=0.01). Pre-incubation of antigen B with the studied metabolites leads to a significant decrease in the mean green pixel intensity in comparison with the control (lactate - 0.2 ± 0.04, p<0.0001, pyruvate - 0.28 ± 0.073, p<0.0001) and the effect of lactate and pyruvate in this case is comparable and indistinguishable from each other. The observed difference in the reaction of antigenic determinants to the addition of intermediates can be explained by the chemical structure: antigens A and B have a common precursor substance - fucose, but differ in the end determinants: in antigen A terminal oligosaccharide is represented by N-acetylgalactosamine, in antigen B by galactose.

**5. Conclusion**

The following conclusions were drawn based on the research conducted:

1. Lactate and pyruvate may participate in protein-protein interactions.
2. With respect to the action on antigenic determinants A and B, the effects of lactate and pyruvate are co-directed, but the effect of lactate is more pronounced than the one of pyruvate.
3. The effect on antigen A is registered as the aggregation process intensification, manifested by an increase in the number of complexes, their area and mean green pixel intensity.

4. In the case of antigen B, the effect of inhibition of antigen-antibody reaction is observed, which is confirmed by a decrease in the number of formed complexes, their area and mean intensity of green pixel.

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