ELSEVIER

Journal of Immunological Methods 252 (2001) 171–174

JIM
Journal of Immunological Methods

www.elsevier.nl/locate/jim

Technical Note

Biotinylation of protein complexes may lead to aggregation as well as to loss of subunits as revealed by Blue Native PAGE

Wolfgang W.A. Schamel *

Department of Molecular Immunology, Biologie III, University of Freiburg and Max-Planck-Institut für Immunbiologie, Stübeweg 51, 79108 Freiburg, Germany

Received 20 December 2000; accepted 10 January 2001

Abstract

Blue Native polyacrylamide gel electrophoresis (BN-PAGE) is a high-resolution method for studying native protein complexes. Here, the migration behaviour of the B cell antigen receptor (BCR) in BN-PAGE with and without prior biotinylation of the cell surface, from which the complexes were purified, are compared. Non-biotinylated complexes appear as a single band, whereas biotinylated complexes display several bands, indicating that biotinylation leads to aggregation of complexes as well as to loss of subunits. Thus, BN-PAGE has limitations in studying multiprotein complexes. These results are used to gain insight into the stoichiometry of the complex. © 2001 Elsevier Science B.V. All rights reserved.

Keywords: Blue Native; Stoichiometry; Biotin; Structure; BCR; Protein complex

Separation of proteins by gel electrophoresis can be performed under native or denaturing conditions. Blue Native polyacrylamide gel electrophoresis (BN-PAGE) is a charge shift method, in which the electrophoretic mobility of a protein is determined by the negative charge of the bound Coomassie dye and the size of the protein. Coomassie does not act as a detergent and was reported to preserve the native structure of oligomeric protein complexes (Schägger et al., 1994). The resolution of BN-PAGE is much higher than that of gel filtration or sucrose density gradient centrifugation (Schägger et al., 1995). Thus, in combination with a second dimension denaturing sodium dodecylsulfate (SDS)-PAGE analysis, the BN-electrophoresis method has been used for the analysis of the molecular mass and composition of protein complexes (Dekker et al., 1997; Schägger et al., 1994; Schamel and Reth, 2000). It has been noted, however, that the molecular weight of certain proteins cannot be deduced from their electrophoretic mobility in BN-PAGE (Moro et al., 1999; Schägger et al., 1994; Schamel and Reth, 2000). This was probably due to either a very basic pI (higher than 9.0), or the failure of the protein/glycoprotein to bind Coomassie.

Abbreviations: BCR, B cell antigen receptor; BN-PAGE, Blue Native polyacrylamide gel electrophoresis; HC, heavy chain; Ig, immunoglobulin; LC, light chain; SDS-PAGE, sodium dodecylsulfate polyacrylamide gel electrophoresis

* Current address: Centro de Biologia Molecular, Facultad de Ciencias, Universidad Autonoma, Cantoblanco, 28049 Madrid, Spain. Tel.: +34-91-397-5074; fax: +34-91-397-4799.

E-mail address: Wschamel@cbm.uam.es (W.W.A. Schamel).

0022-1759/01/$ - see front matter © 2001 Elsevier Science B.V. All rights reserved.
PII: S0022-1759(01)00357-X

After their separation by PAGE, proteins can be visualized by immunoblotting, using specific antibodies or by general stains as Coomassie or silver procedures. Alternatively, proteins can be biotinylated before their separation and detected by Western blotting using streptavidin. Commonly used protocols include biotinylation of proteins on the surface of living cells prior to cell lysis (Kim et al., 1994) or biotinylation of proteins after their purification (Kim et al., 1994; Schamel, 1999).

Here, the B cell antigen receptor (BCR) complex was used as a model system to investigate its electrophoretic mobility in BN-PAGE, with and without prior biotinylation. The BCR consists of the disulfide-linked Ig-α/Ig-β heterodimer and the disulfide-linked membrane-bound immunoglobulin (mIg) molecule, which is composed of two heavy chains and two light chains (HC_2LC_2) (Reth, 1992). Previously, a dual affinity method to purify the BCR from digitonin lyzed cells was developed and it was shown that in BN-PAGE, it runs as a single band of 500 kDa (Fig. 1A and Schamel and Reth, 2000), which is larger than the expected molecular weight of 300 kDa. If SDS was added to the sample prior to its separation, in order to disrupt the BCR complex, the mIgM molecule can be seen at 280 kDa (Fig. 1A). Next, the BN-PAGE-separated BCR complex (without SDS) was applied to second dimension SDS-PAGE and Western blot analysis. Development of the Western blot with anti-HC, anti-Ig-β antibodies, anti-Ig-α and anti-LC antibodies (Fig. 1B) showed that all the components are present in the BN-PAGE-separated BCR. These data clearly demonstrate that the digitonin-solubilized BCR is a complex of defined size.

If the purified IgM–BCR was biotinylated before its separation by BN-PAGE, it appears in five different size forms (Fig. 2A). A second dimension SDS-analysis (Fig. 2B) showed that the fastest migrating form (300 kDa) was the mIgM molecule detached from the Ig-α/Ig-β heterodimer, whereas all the other forms contained the mIgM and Ig-α/Ig-β molecules. From comparison with Fig. 1A, it is concluded that the second fast migrating form (500 kDa) corresponds to one IgM–BCR complex, and the higher forms (900, 1700 and 3000 kDa) correspond to aggregated mIgM/BCR complexes. Disrupting these forms by SDS prior to their separation

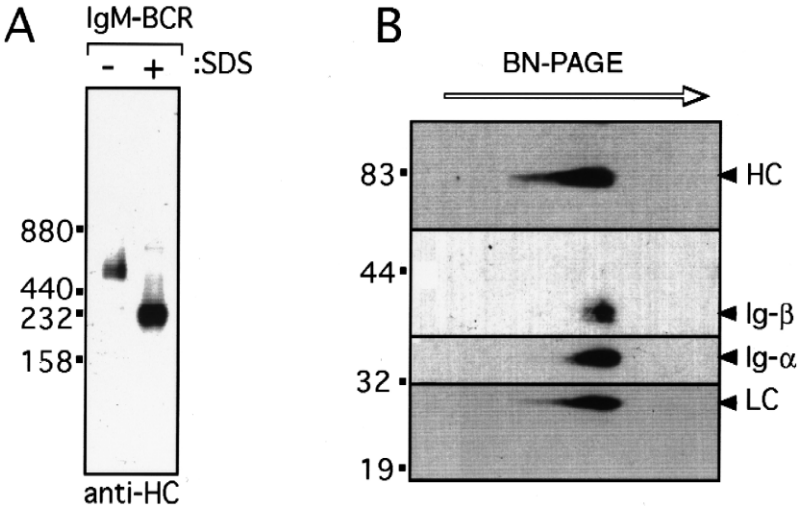

Fig. 1. Size separation of a BCR complex by Blue Native PAGE. (A) The IgM–BCR was purified from J558Lμm/Ig-α cells by a two-step affinity chromatography (Schamel and Reth, 2000) and analyzed by BN PAGE (Schägger et al., 1994) in the absence (−) or presence (+) of 1% SDS. Immunoblotting was done with a horseradish peroxidase (HRPO)-coupled anti-HC (anti-μ) antiserum (Southern Biotechnology) and the ECL system (Amersham). (B) The purified IgM–BCR was size-separated by BN-PAGE (first dimension), and after reduction, further analyzed by SDS-PAGE (second dimension). Probing of the membrane with the indicated antibodies allowed identification of the individual components: anti-HC antiserum (see above), monoclonal anti-Ig-β antibodies, anti-Ig-α antiserum (kind gift of Dr. J. Jongstra) and HRPO-coupled anti-LC (anti-λ) antiserum (Southern Biotechnology).

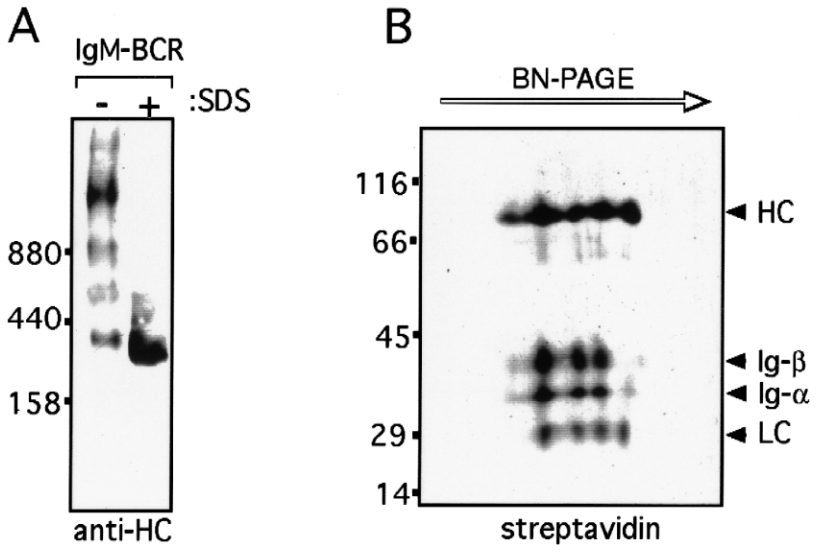

Fig. 2. Size separation of a biotinylated BCR complex by Blue Native PAGE. (A) The purified IgM–BCR was biotinylated with 1 mg/ml sulfo-NHS-biotin (PIERCE) for 30 min at 4°C before its elution from the column and size-separation by BN-PAGE in the absence (−) or presence (+) of 1% SDS. Immunoblotting was done with an anti-HC antiserum (see above). A similar result was obtained with HRPO-coupled streptavidin (not shown). (B) The biotinylated IgM–BCR was separated by BN-PAGE (first dimension), and after reduction, further analyzed by SDS PAGE (second dimension). Probing of the membrane was done with HRPO-coupled streptavidin (Amersham).

by BN-PAGE showed that biotinylated mIgM has an electrophoretic mobility similar to the non-biotinylated mIgM molecules (Figs. 1A and 2A), indicating the the individual subunits of the complex did not alter their mobility if biotinylated.

The biotinylated IgM–BCR complex appeared in several size forms, of which, one had lost the Ig-α/Ig-β subunit (Fig. 2). Since the non-biotinylated receptor complex was only found in one size form (Fig. 1 and Schamel and Reth, 2000), the biotinylation procedure generated artefacts that were detected by BN-PAGE. The loss of a subunit may be due to the biotinylation of amino acids implicated in the binding of this subunit to the rest of the complex. On the other hand, biotinylation may lead to new interactions generating non-covalent oligomers of the receptor. Although Coomassie binds to hydrophobic protein surfaces and reduces aggregation, the biotinylated BCRs may have aggregated during their separation by BN gels. Alternatively, aggregation may have happened before running of the gel. The same results were obtained with the IgD–BCR, whereas biotinylation of bovine serum albumin did not lead to aggregation (data not shown). Similarly, the monoclonal antibodies M2 (anti-flag tag, Kodak) or 12CA5 (anti-HA tag, Boehringer) did not aggregate upon biotin or fluorescein conjugation. To prevent these artefacts, already size-separated protein complexes can be biotinylated within the gel, using the biotinylation in gel (BIG) method (Schamel, 1999), or after their transfer to a membrane (LaRochelle and Froehner, 1986; Schamel, 1999). The BIG method is considered by the author as the best choice, since it is the most sensitive general protein stain available to date.

Importantly, biotinylation of the BCR also yielded useful information. Only one complex smaller than the IgM–BCR was found, namely free mIgM. The same observation was made with biotinylated IgD–BCRs (data not shown). This indicates that the BCR (mIg:Ig-α/Ig-β) complexes might only posses one Ig-α/Ig-β heterodimer, since otherwise, two smaller bands would have been found that corresponded to the loss of one or two Ig-α/Ig-β dimers. Indeed, it is recently shown that the IgM, as well as the IgD–BCR, have a 1:1 stoichiometry (mIg:Ig-α/Ig-β, Schamel and Reth, 2000) and not a 1:2 stoichiometry.

Here, it is shown that covalent modification of protein complexes (e.g. by biotin) may lead to an artefact in subunit composition and oligomeric state, which might give clues to the stoichiometry of a multiprotein complex.

Acknowledgements

The author would like to thank Dr. Michael Reth for his support and for reading the manuscript. This work was supported by the Deutsche Forschungsgemeinschaft through SFB 388 and the Leibniz Prize to Michael Reth.

References

Dekker, P.J.T., Martin, F., Maarse, A.C., Boemer, U., Mueller, H., Guiard, B., Meijer, M., Rassow, J., Pfanner, N., 1997. The Tim core complex defines the number of mitochondrial translocation contact sites and can hold arrested preproteins in the absence of matrix Hsp70–Tim44. EMBO J. 16, 5408.

Kim, K.-M., Adachi, T., Nielsen, P.J., Terashima, M., Lamers, M.C., Köhler, G., Reth, M., 1994. Two new proteins preferentially associated with membrane-immunoglobulin D. EMBO J. 13, 3793.

LaRochelle, W.J., Froehner, S.C., 1986. Immunochemical detection of proteins biotinylated on nitrocellulose replicas. J. Immunol. Methods 92, 65.

Moro, F., Sirrenberg, C., Schneider, H.-C., Neupert, W., Brunner, M., 1999. The TIM17.23 preprotein translocase of mitochondria: composition and function in protein transport into the matrix. EMBO J. 18, 3667.

Reth, M., 1992. Antigen receptors on B lymphocytes. Annu. Rev. Immunol. 10, 97.

Schägger, H., Cramer, W.A., von Jagow, G., 1994. Analysis of molecular masses and oligomeric states of protein complexes by blue native electrophoresis and isolation of membrane protein complexes by two-dimensional native electrophoresis. Anal. Biochem. 217, 220.

Schägger, H., Brandt, U., Gencic, S., von Jagow, G., 1995. In: Attardi, G.M., Chomyn, A. (Eds.), Methods in Enzymology vol. 260, 82.

Schamel, W.W.A., 1999. Detection of proteins after biotinylation within polyacrylamide gels (BIG method). Anal. Biochem. 274, 144.

Schamel, W.W.A., Reth, M., 2000. Monomeric and oligomeric complexes of the B cell antigen receptor. Immunity 13, 5.