Micellar enzymology: methodology and technique

A. V. Levashov and N. L. Klyachko

Department of Chemistry, M. V. Lomonosov Moscow State University, Leninskie Gory, 119899 Moscow, Russian Federation. Fax: (095) 939 3429. E-mail: klyachko@enzyme.chem.msu.ru

The methodological procedures employed both for serving investigations in the field of micellar enzymology and developed directly in micellar enzymology and being of general significance, such as an estimation of molecular masses and sizes of biocatalysts, titration of active sites of enzymes, chemical modification of proteins (enzymes), conjugation and nanogranulation, are reviewed. Potentialities of using and informativity of various techniques are analyzed.

Key words: reverse micelles, proteins, enzymes, structure and function, methods for research, micellar enzymology.

Studies of proteins (enzymes) in systems of reverse micelles (or, more generally, in three-component systems water—surfactant—organic solvent) are being intensely carried out for more than 30 years and have resulted to the moment in the formation of a broad independent area of knowledge named "micellar enzymology." The progress and state-of-the-art of research in this area can be traced from reviews.1−12

In addition to a rich fundamental material, which enlarge concepts about the nature of enzymes and biocatalysis, the powerful methodical basis has been created for serving and development of studies in the field of micellar enzymology. This contains novel methods for manipulation with proteins (enzymes) and revealing their characteristics. Among them are new methods for the isolation and purification of proteins (enzymes), their chemical modification, titration of active sites, and designing of complexes, conjugates, and supramolecular ensembles. In the present work, it is to this methodical and technical aspects of micellar enzymology that the main attention is given.

First, note that unlike traditional homogeneous aqueous solutions, micellar systems in organic solvents are microheterogeneous (pseudohomogeneous), although they remain completely transparent and suitable for studying by classical instrumental methods. In other words, micellar systems combine the properties of standard homogeneous solutions* and particles, viz., micelles, which exist in dynamic equilibrium. During solubilization, enzymes are incorporated into micelles, and it is micelles (micellar matrix) that are the basic factor of action upon, and regulation of, solubilized enzymes.

1. The main experimental methods

1.1. Procedures for the incorporation of proteins (enzymes) into reverse micelles

Proteins (enzymes) can be incorporated into reverse micelles of surfactants in organic solvents using one of three following methods.

The first method, the so-called "injection,"13 is presently used most widely. Small amount (several percent v/v) of an aqueous solution of a protein is introduced into a solution of a surfactant in an organic solvent (anhydrous or little hydrated). The particular ratio of the amounts of the aqueous solution to the organic one is determined by experimental conditions, first, by the value of the required degree of hydration of the surfactant (\(w_0 = [\text{H}_2\text{O}] / [\text{Surf}]\)). The resulting mixture is vigorously shaken (for seconds or tens of seconds) until an optically transparent solution is formed. This method is in fact simple and efficient. Some problems appear when it is used, such as the questions about the state of equilibrium in the system obtained (see, e.g., Ref. 14), processes and reactions that occur during the preparation of the system. The question about equilibrium can be answered by comparison with other methods for the achievement of this state (since the equilibrium state is independent of the way of its achievement). Some undesirable side reactions (leading, in particular, to partial inactivation of the solubilized enzyme) can be excluded by selection of the order of adding and mixing of the components. For example, solutions of the enzyme and substrate with the same final degree of hydration are prepared separately and incubated (equilibrated) for some time. Then the reaction is initiated by mixing of the stored solutions in a required proportion (we specially emphasize that in this

* Micellar systems are diphilic in nature and allow the operation with both hydrophilic (water-soluble) and hydrophobic (soluble in organic solvents) components.
case the degree of hydration of the surfactant, which is often the critical factor in the manifestation of the nonequilibrium character and side impeding phenomena, is not virtually changed in the final stage, viz., initiation of the enzymatic reaction).

The second method proposed by Menger and Yamada\textsuperscript{15} consists, first, in addition of the required amount of water (an aqueous buffer solution) to a solution of a surfactant in an organic solvent for the desired degree of hydration ($w_o$) to be achieved followed by dissolution of a dry (for example, lyophilized) protein preparation in the resulting micellar solution with vigorous shaking (stirring). The time required for the dissolution of dry protein is usually much longer than that for the solubilization of aqueous solutions (from several min to tens of h). In this procedure, the protein exists for a comparatively long time in contact with the surfactant and organic solvent and, as a rule, it is partially denatured. However, micellar solutions with a higher concentration (up to the saturation concentration) of the protein compared with that achieved in the first (injection) method can finally be obtained (with some loss of the protein). To decrease the protein losses, it is reasonable to perform dissolution in several stages: first, a small excess of the dry protein is taken; after several h of stirring of the suspension, the supernatant is separated, a new portion of the dry protein is added, the dissolution procedure is multiply repeated, and the content of the dissolved protein is determined in the supernatant. When using this procedure, one should keep in mind that a partial loss of the surfactant and water because of precipitation can occur during the formation of the system. This circumstance needs to be additionally monitored, e.g., by chromatography.\textsuperscript{16} The surfactant concentration can easily be determined in a micellar solution due to its high content, for example, gravimetrically from the mass of the dry residue after the evaporation of the solvent from an aliquot of the analyzed solution. The water content in micellar system can conveniently be monitored by $^1$H NMR using the chemical shift value.\textsuperscript{17} In discussion of problems of control of the water content, note the problem of characterizing the hydration of the starting surfactant preparations (the latter should be taken into account for the correct calculation of the working degrees of hydration). With this purpose, we successfully used IR spectroscopy and monitored the water content at a frequency of 3420 cm$^{-1}$ (the procedure is described in detail in Ref. 18).

It should be emphasized that when the second method of solubilization (with the dissolution of a dry protein preparation in a micellar solution) is used, the content of both protein and low-molecular-weight (in particular, salts) admixtures can vary in the resulting solution. The third method\textsuperscript{19–21} is based on the spontaneous transfer (distribution) of protein in a biphasic system composed of approximately equal volumes of an aqueous solution of the protein and an organic solvent containing the surfactant (the micellar system with a specified degree of hydration). The protein transfer occurs without stirring or with gentle stirring and takes a rather long time (from tens of min to days). During this time interval, the protein (enzyme) actively interacts with molecules of the surfactant and the organic solvent, it is concentrated at the interface and undergoes denaturation. This method is inferior to those described previously because of being labor-consuming and being accompanied by the difficulties indicated. On the other hand, the method discussed has an independent significance and, undoubtedly, is very promising for the separation and purification of proteins (see, e.g., Ref. 22) because such processes as the incorporation of protein molecules into micelles and exit from micelles to an aqueous solution can conveniently be controlled by the variation of such parameters as pH and ionic strength. Conditions for the selective incorporation into micelles can be found for each protein, and the desired protein can thus be extracted from a mixture. Replacing an aqueous phase by a pure solution and establishing the required pH and ionic strength values, re-extraction is carried out, and a solution of the target (purified) protein in water is obtained. This procedure can also be used for the extraction of other water-soluble components, including reaction products, from micellar systems. This will be discussed in the next Section.

1.2. Separation of components of micellar systems, regeneration of the enzyme, and isolation of reaction products

Since micellar systems are microheterogeneous, it is rather difficult to isolate one or another component from them. This problem, most likely, has no general solution, and each particular case requires individual consideration. At the same time, the whole set of approaches to the solution of this problem is available, and we are going to consider it successively.

Both the simplest and the most elegant method is the reversible separation of phases under physical action. For example, upon a relatively slight temperature change (in some case, with the temperature increase, and in other cases, with its decrease),\textsuperscript{23} the phase which is rich in the organic solvent and contains the hydrophobic product and almost no surfactant, is separated from the micellar solution. This method seems to be most promising, technologically acceptable, and appropriate for purposes of organic synthesis where the product can be obtained in the pure state by the evaporation of the organic solvent and the initial system is easily recovered by addition of the pure organic solvent.

A general method for the separation of a low-molecular-weight component from micellar systems without their destruction is the use of semipermeable membranes. This principle can form the foundation for designing continuous reactors.