*Pure & Appl. Chem.*, Vol. 51, pp. 1405-1407.
Pergamon Press Ltd. 1979. Printed in Great Britain.

# THE EFFECT OF POLYMERIC MATRIX ON THE FUNCTIONS AND PROPERTIES OF IMMOBILIZED ENZYMES

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Immobilized enzymes provide today a powerful tool for development of new chemical and biochemical processes in inductry. Due to the fact that some enzymes are quite expensive, the stability of them in an immobilized form is of importance. Immobilization of a given enzyme in some cases increases its stability, but to forecast this effect is very difficult. In order to gain some new information, a systematic study has been performed in Moscow University. We have started with a simple approach that incorporation of an enzyme molecule into a dense polymeric matrix should prevent unfolding of the tertiary structure of a protein. The result of the experiments with chymotrypsin, which has been entrapped in cross-linked polimethacrylate gel, confirmed this suggestion /1/. The dependence of the first order rate constant of thermoinactivation of chymotrypsin (60°C, pH 8.0) upon gel concentration has been investigated. It turned out that after gel concentration has reached about 25%, very strong increase in the enzyme stability occured. At a 50% gel concentration, the stability of the enzyme is $10^5$ times higher than in solution. The experiments with the light depolarisation and the kinetic measurements showed that stabilisation effect is a direct result of enzyme "freezing" in a polymeric matrix. At 25% gel concentration translational diffusion of the enzyme molecules in the matrix is arrested and at 40% gel concentration rotational diffusion is arrested /1/. These facts suggest the so called "multipoint interaction" model of enzyme stabilisation. According to this model, an enzyme molecule entrapped in a matrix of gel, which contains functional groups like COOH, establishes week bonds (e.g. hydrogene bonds) with the elements of the matrix structure. Due to the density of the matrix, the surroundings of these bonds reinforces the strength of the protein molecule and increases its stability /1/. To prove this model, the following experiment has been performed. Chymotrypsin has been succinylated in such a way that, instead of the $NH_2$ groups, on the surface of the enzyme molecule charged carboxylic groups appear. This chymotrypsin, additionaly modified by a dansyl group, has been entrapped in polymethacrylate gel of various concentration and the apparent relaxation time of the enzyme molecule has been measured. Up to 50% gel concentration, the relaxation time remains the same as in water. In other words, in this case the freedom of enzyme rotation is not influenced by its surroundings due to the fact that deprotonated carboxylic groups of the modified enzyme suffer a strong repulsion with respect to the negatively charged carboxylic groups of the gel. Likewise, the stability of this modified enzyme is not changed in the same range of gel concentration. It showes that the enzyme-matrix interaction and stability of the enzyme are very closely related /1/. Similar results have been obtained with nonmodified chymotrypsin entrapped in neutral polyacrylamide gel /1/. In case of chymotrypsin immobilized in polymethacrylate gel only week bonds exist between the enzyme molecule and the matrix. It seems natural that if these bonds are covalent the effect of stabilisation will also be observed and, possibly, to a greater degree. In order to provide covalent bonding between the entrapped enzyme and the matrix, vinyl groups have been introduced to the surface of the enzyme molecule. For this purpose, chymotrypsin was treated by acryloyl chloride, and modification of different amounts of amino groups was accomplished. Then copolymerization of modified chymotrypsin with methacrylate or acrylamide and cross-linking agents has been performed. The final product retains most of the activity of the introduced chymotrypsin. The copolymerized chymotrypsin has been tested for thermostability which turned out to be strongly dependent on the amount of the introduced vinyl groups and almost completely independent of the gel concentration in the range of 8 - 47% /2/. These and some other facts (see /2/) suggest the following mechanism of stabilisation. The enzyme molecule during copolymerization establishes strong covalent bonds with the three-dimensional network of the gel polymeric

chains. This network is sufficiently rigid to reinforce the rigidity of the enzyme structure and provide a certain amount of stability. The greater number of bonds is formed between the enzyme and the moleuclar network of the gel, the stronger is this effect. It seems that the gel concentration in the immediate environment of the entrapped enzyme is determined solely by the number of the vinyl groups on the surface of the enzyme molecule. This accounts for the fact that the enzyme stability does not depend on the gel concentration.

The reinforcement of the enzyme structure can be achieved in a different way. Let us take an enzyme molecule and surround it using long polymeric molecule which has side groups capable of forming different kinds of bonds with the functional groups of the enzyme surface. In the experiments of this kind, use was made of formate dehydrogenase of bacterial origin and a copolymer of vinylpyridine and acrolein. The pyridinium side groups were partially alcylated by treatment with methyliodide. The resulting polymer contains following side groups: positively charged N-methylpyridinium, aldehyde and hydrophobic pyridinium groups.

Formate dehydrogenase treated by this polymer remains soluble, but its stability increases very considerably /3/. For instance, the half time of decay of the soluble enzyme is about 30 hours, the half time of decay is over 1000 hours. It has been proved experimentally that this kind of treatment gives rise to formation of very tight compound with an enzyme-polymer stoichiometry of 1:1, due to formation of covalent bonds (Shiff bases), ion pairs (carboxylic groups and methylpyridinium groups) and hydrophobic linkages (pyridinium grous). The final shape of the modified enzyme can be visualized as a protein globule wound around by a polymer molecule.

This type of stabilisation is of interest in the cases when a stable but soluble preparation is required. Although it is necessary to keep in mind that the specificity of such an enzyme can strongly differ from the original material.

Among the properties of enzymes entrapped in polymeric matrices, of special interest are peculiarities of electrochemical processes in which an immobilized enzyme can take part. This phenomenon is a detail of a more general process which has been recently christened as "Bioelectrocatalysis". In general, bioelectrocatalysis deals with conjugation of biochemical and electrode reaction Systems of reactions are in some instances very complicated, but recognition of biocatalytic contribution to the general process does not represent any difficulty.

For a simplest case the following simplified scheme is very often valid /4/

$$E + S \rightleftharpoons ES \longrightarrow E\ (\pm n\bar{e}) + P$$

$$E\ (\pm n\bar{e}) \xrightarrow{\text{electrode}} E \pm n\bar{e}\ (\text{electrode})$$

Here E ($\pm n\bar{e}$) stands for the enzyme active centre which is in an oxidized or reduced form. The overall result of these consecutive processes is reduction or oxidation of the corresponding substrates and generation of energy in the form of the electrode potential.

Owing to the high activity of the enzyme the limiting step in this system is usually an electrode reaction. There are two possibilities to facilitate this process. First, use can be made of low molecular weight mediators. Mediators have to be good substrates for a given enzyme which could undergo rapid electrochemical conversion on the electrode. For instance, hydrogen cannot be oxidized on a graphite electrode at a normal potential. But if hydrogenase (enzyme) is immobilized on an electrode surface, and a mediator (methyl viologen) is introduced to the system, then hydrogen will undergo smooth oxidation at a reversible potential /5/. The sequence of events is as following:

$$E + H_2 \rightleftharpoons EH^- + H^+$$

$$EH^- + 2MV^{2+} \rightleftharpoons E + 2MV^+ + H^+$$

$$2MV^2 \rightleftharpoons 2MV^{2+} + 2\bar{e}\ (\text{electrode})$$

The net reaction is oxidation of molecular hydrogen and generation of a potential on electrode. This system can be regarded as a hydrogen fuel electrode /5/

Despite of good results which can be obtained with the use of mediators, there are some drawbacks in this case. Side reactions which might eventually destroy the mediator is the major ones. In order to avoid this attempts were made to create a system in which direct transport of electrons between the enzyme active centre and an electrode is possible.

For this purpose, use was made of a conductive polymeric matrix in which a hydrogenase of microbial origin had been entrapped /6/. A complex formed by 1-polypropargylpyridinium and tetracyanochinodimethane was used as a matrix for immobilization of hydrogenase. The entrapment capacity of this matrix is very high. It is possible to immobilize up to 20% of hydrogenase (by weight) in this non-soluble complex. If this enzyme containing complex is brought into contact with a graphite electrode, hydrogen readily undergoes ionization at a reversible electrode potential /6/.

This approach is also valid in the case of an oxygen electrode based on the use of laccase kindly provided to us by prof. B. Malmström (Geteborg Univ.). Laccase immobilized on a carbon surface is able to pick up electrons from an electrode and to chanel them to oxygen molecules reducing these into water /7/.

In all described cases the intimate mechanism of the direct electron transfer between the enzyme active centre and a polymeric matrix or polymeric support is still to be understood. But is is evident that this phenomenon may play a very important role in many biocatalytic and bioelectrocatalytic systems.

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