CHEMICAL GRAFTING OF FUNCTIONAL NAD IN THE ACTIVE SITE OF A DEHYDROGENASE REGENERATION IN SITU

Marie-Dominique LEGOY, Jean-Michel LE MOULLEC and Daniel THOMAS

Laboratoire de Technologie Enzymatique. ERA no. 338 du CNRS, Université de Technologie de Compiègne, BP 233, 60206 Compiègne, France

Received 3 August 1978

1. Introduction

Immobilization and stabilization of enzyme systems by various methods [1] have increased the possibilities in enzyme applications. One of the limiting steps in the further development of enzyme use is the regeneration of cofactors, especially the pyridine nucleotide cofactors which are involved in a large number of metabolic pathways. A soluble high molecular weight derivative of NAD has been used [2] which can be retained in a continuous flow reactor by an ultrafiltration membrane. Soluble immobilized NAD in an enzyme electrode has also been applied [3]. Electrochemical regeneration of NAD from its reduced form [4], cofactor recycling in liquid membrane–enzyme system [5], and recycling of NAD within semi-permeable aqueous microcapsules containing a multienzyme system [6] have been described. High turnover NAD regeneration in the coupled dehydrogenase conversion of sorbitol to fructose has been studied [7]. A Sepharose alcohol dehydrogenase–NAD complex requiring no addition of soluble cofactors has been reported [8]. The system, based on a substrate-coupled regeneration, gives a solution for both problems of retention and regeneration but such preparations are limited in their use to a small number of reactions.

This paper deals with the immobilization of the cofactor at the active site of an enzyme and its regeneration with oxygen using phenazine methosulfate (PMS) as an electron carrier. In this technique alcohol dehydrogenase (ADH) and NAD were immobilized together in a protein membrane.

2. Materials and methods

Membrane production was done by the crosslinking method in [9]. From a solution containing 0.5 mg/ml baker's yeast ADH, 2.5 mg/ml NAD, 44 mg/ml albumin, 4.5 mg/ml glutaraldehyde in 0.02 M phosphate buffer, (pH 6.8) 2 ml was spread on a flat glass plate. After 12 h complete insolubilization occurred and a membrane was produced. The membrane area and thickness was 30 cm^2 and 50 μm, respectively. The enzyme membrane was thoroughly rinsed with a 0.02 M phosphate buffer (pH 6.8), until the rinse solution no longer showed A_{280} values.

Enzyme activity, in absence of cofactor regeneration, was spectrophotometrically measured at 340 nm in a solution of 0.1 M pyrophosphate-glycine buffer (pH 8.5), containing 3 mg/ml NAD and 20 mg/ml ethanol.

The enzyme cofactor membranes were tested with a CLARK pO_2 electrode (E 5040 Radiometer). The active membrane was fixed together with the gas selective membrane onto the electrode jacket.

The enzyme electrode was tested in a continuous flow device in which a solution of 0.1 M pyrophosphate–glycine buffer (pH 8.5), containing 0.02 mg/ml PMS was introduced into the solution just prior to contact with the enzyme.

An electrode having a membrane containing enzyme but no NAD was used as a reference in order to check the differential pO_2 value. The reaction medium was supplied with oxygen by bubbling air through it. After leaving the active part of the device, the solution flowed through a 12 cm column containing cross-

linked albumin. The PMS was quantitatively adsorbed in the packed bed and was no longer detectable at 260 nm in the solution leaving the apparatus.

The fluorescence analysis of the enzyme membrane was done with a spectrofluorimeter JY3 (Jobyn-Yvon, ISA). The exciting wavelength was 375 nm and the emitted light was measured at 460 nm. The enzyme cofactor membrane was maintained in the right position with a frame work inside the cuvette. The angles of the membrane with exciting and emitted beams are of 45°.

Alcohol and PMS were added in the cuvettes without moving the studied membrane.

3. Theoretical note

The equations for oxygen and substrate in the active coating may be written:

$$D_S \frac{d^2S}{dx^2} = V_m f(S,O_2) \quad (1)$$

$$D_{O_2} \frac{d^2O_2}{dx^2} = V_m f(S,O_2) \quad (2)$$

From (1) and (2) $D_S (S_o - S_e) = D_{O_2} (O_{2o} - O_{2e})$

where S_o = concentration in the bulk solution and S_e = concentration along the sensor under zero order kinetics for both substances.

$$V_m = \left[2(S_o - S_e) D_S\right] / e^2 \quad (3)$$

$$V_m = \left[2 \frac{D_S^2}{D_{O_2}} (O_{2o} - O_{2e})\right] / e^2 \quad (4)$$

The enzyme activity is easily obtained from (4).

4. Results

The resulting enzyme activity as a function of the NAD concentration during immobilization is given in fig.1. Maxima are observed for both tight and open structures.

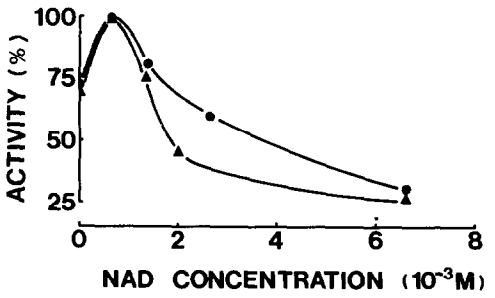

Fig.1. Activity yield as a function of NAD concentration during immobilization. The value of 100 is given to the maximum resulting activities (28% and 13%, compared to the native enzyme) for tight (–▲–) and open (–●–) structures, respectively.

An active site protection by the cofactor occurs for NAD at $< 6 \times 10^{-4}$ M. At $> 2 \times 10^{-3}$ M the activity is lower than that observed without any added cofactor. The phenomenon is not observed when deamino NAD is used in place of NAD (fig.2). The apparent inhibition by excess NAD during immobilization can be explained by an immobilization of the cofactor within the active site of the enzyme. This can be checked by chemical regeneration of the bound NAD in situ. The enzyme activity, without free NAD in the bulk solution, was shown by using an electrode method.

Enzyme electrodes are not only good tools from the applied point of view but are also useful from a fundamental point of view for kinetic studies of artificial enzyme membranes. Kinetic measurements in a

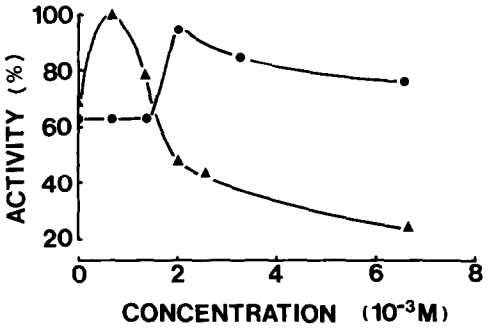

Fig.2. Activity yield as a function of the cofactor concentration during immobilization. Activities are given for membranes of 30 cm² containing either NAD (–▲–) or deamino NAD (–●–).

bulk solution, for example in a batch reactor, give information dealing with the overall system. An electrode system gives kinetic information dealing only with the insoluble phase and artifacts, such as enzyme and cofactor leakage are eliminated. Due to the stationary state established between the enzyme reaction and diffusion, an electrode device is able to transform a reaction rate into an absolute value as a local concentration. An electrode bearing the enzyme cofactor membrane can be tested in a solution without additional free NAD. A second electrode bearing the same coating, but without NAD, introduced in the same solution is a permanent blank during the experiments. With this device the differential pO_2 measured gives the enzyme activity directly (see section 3). It is possible to record the enzyme activity directly as function of time. A transient state behavior of such an electrode is given in fig.3. There is experimental evidence for ADH activity with O_2 consumption in the absence of any free NAD. The response given by the reference electrode shows beyond doubt an intra-membrane phenomenon.

The continuous use of the membranes is possible and the PMS can be removed from the reaction medium by adsorption in a column containing crosslinked albumin.

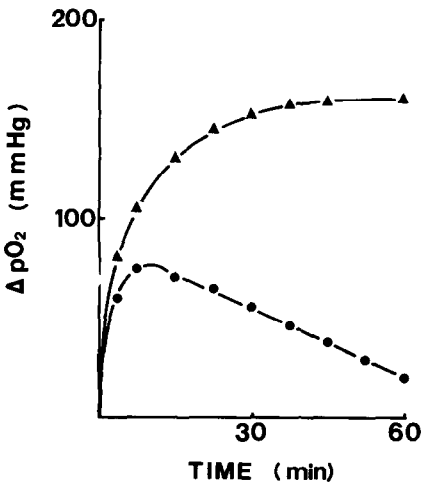

Fig.3. Differential pO_2 measured as a function of time during the transient state. Measurements were taken with a pO_2 electrode coated with a membrane bearing an immobilized NAD–ADH system (–▲–). There was no free NAD in the solution and the PMS was 0.02 mg/ml. The response of an electrode bearing an ADH coating (without NAD) immersed in the same solution is given as a reference (–●–).

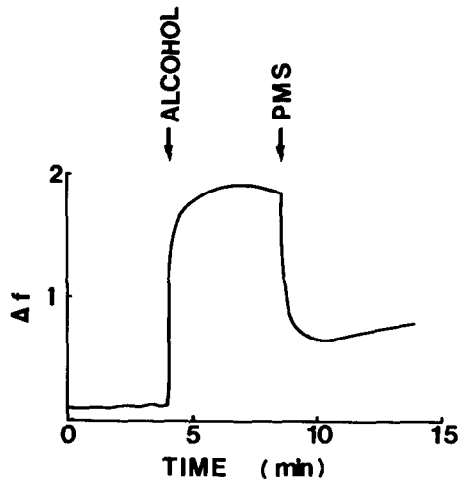

Fig.4. Enzyme–NAD membrane: change in fluorescence (Δf) as a function of time after introducing alcohol and PMS (details in text).

The membrane was also tested in spectrofluorimetry (fig.4). When alcohol is introduced in contact with the enzyme–NAD membrane a dramatic increase of intensity of the emitted light was observed. A decrease was shown after introducing PMS. With an enzyme membrane without immobilized NAD no variation of the intensity of the emitted light was observed. The change in fluorescence (Δf) was studied as a function of the NAD concentration during immobilization process (fig.5).

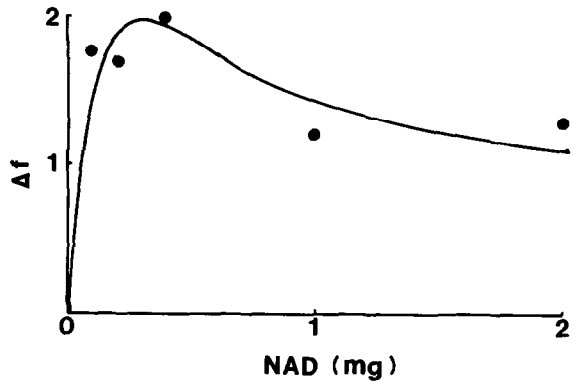

Fig.5. Enzyme–NAD membrane: change in fluorescence after introducing alcohol as a function of the NAD concentration during the immobilization process.

5. Conclusion

A functional NAD molecule was immobilized at the active site of Alcohol dehydrogenase within a proteic membrane. The presence and the functionality of the cofactor was checked by fluorescence analysis. The dehydrogenase NAD membrane does not require addition of soluble cofactor for its activity. The system represents a new worthwile approach because both problems of retention and regeneration of cofactor are solved. The method can be used not only for industrial and analytical applications but also to try to get a better understanding of the kinetics and mechanisms of the catalytic action of dehydrogenase.

References

[1] Mosbach, K. (1976) Methods Enzymol. 44.
[2] Wykes, J. R., Dunnill, P. and Lilly, M. D. (1975) Biotechnol. Bioeng. 17, 51.
[3] Davies, D. and Mosbach, K. (1974) Biochim. Biophys Acta 370, 329.
[4] Coughlin, R. W., Aizawa, M., Alexander, B. F. and Charles, M. (1975) Biotechnol. Bioeng. 17, 515
[5] May, S. W. and Landgraff, L. M. (1976) Biochem. Biophys. Res. Commun. 68, 786.
[6] Campbell, J. and Chang, T. M. S. (1976) Biochem. Biophys. Res. Commun. 69, 562.
[7] Chambers, R. P., Walle, E. M., Baricos, W. H. and Cohen, W. (1978) in: Enzyme Engineering (Pye, E. K. and Weetall, H. H. eds) p. 363, Plenum Press, New York.
[8] Gestrelius, S., Mansson, M. O. and Mosbach, K. (1975) Eur. J. Biochem. 57, 529.
[9] Thomas, D., Bourdillon, C., Broun, G. and Kernevez, J. P. (1974) Biochemistry 13, 2995.