BIOSPECIFIC INTERACTION ON SOLID PHASE BETWEEN ALCOHOL DEHYDROGENASE AND AN IMMOBILIZED NADH ANALOGUE STUDIED BY FLUOROMETRY

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1. Introduction

Affinity chromatography has now become a widely accepted technique for the purification of enzymes and other biological macromolecules [1]. Nevertheless, little is known about the various parameters involved in the binding process per se on solid phase. For instance, does the enzyme interact with its immobilized complementary ligand by biospecific interaction or do non-specific factors such as ionic and hydrophobic interactions play a major role [2]? Different methods have been employed, especially in the area of general ligand affinity chromatography [3], to investigate whether and to what extent biospecific adsorption is involved in the affinity-binding step. However, to the authors' knowledge direct spectroscopic measurements of the affinity-bound enzyme in order to obtain information on the biospecific interaction in situ have not yet been performed.

In this communication, we wish to present a novel and direct approach, based on fluorometry, to study enzyme—coenzyme interaction on solid phase between horse liver alcohol dehydrogenase (HLADH) and the immobilized NADH analogue, N^6-[N-(6-aminohexyl)-carbamoylmethyl]-NADH. This technique is similar to that for the study of conformational changes taking place in immobilized enzymes [4,5].

2. Materials and methods

2.1. *Materials*

Alcohol dehydrogenase from horse liver (HLADH)

and from yeast (YADH), β-NADH, β-NAD$^+$ and iodoacetic acid (twice recrystallized from petroleum ether) were obtained from Sigma (St Louis, MO). Sepharose 2B was purchased from Pharmacia (Uppsala) and isobutyramide (twice recrystallized from water) from Eastman (Rochester, NY). Iodo[2-^{14}C]acetic acid (57 mCi/mmol) was purchased from Amersham (England) and 2,5-diphenyloxazole (PPO) and 1,4-bis[2-(4-methyl-5-phenyloxazoylyl)]benzene (dimethyl POPOP) from Araphahoe Chemicals (Boulder, CO). All other chemicals were of reagent grade and used without further purification.

2.2. *Preparation of* N^6-[N-(6-aminohexyl)carbamoyl-methyl]-NADH

N^6-[N-(6-Aminohexyl)carbamoylmethyl]-NAD$^+$ [6] was enzymically reduced with YADH as in [7]. This reduced nucleotide-analogue was more than 95% pure as judged by thin-layer chromatography analysis [6] and exhibited an A_{340}/A_{266} ratio of 0.34.

2.3. *Preparation of NADH—Sepharose*

Sepharose 2B was activated with cyanogen bromide [8]. To 20 ml weakly activated gel (prepared by using 2.5 mg CNBr/ml settled gel) were added 6.5 mg NADH analogue in 30 ml 0.1 M NaHCO$_3$, pH 9. After coupling overnight, at 4°C, with gentle agitation, the gel was washed on a glass filter following the scheme: 0.5 litre 0.1 M NaHCO$_3$, pH 9, 1 litre 1 M NaCl and 200 ml 0.05 M Tris—HCl, pH 9. All preparations were stored in the latter buffer at 4°C. Prior to use, they were washed exhaustively with 1 M NaCl and 0.1 M sodium phosphate buffer, pH 7.5. Activated Sepharose, which had been treated exactly

167

as above except that no ligand had been added was used as a reference gel.

The amount of nucleotide bound was estimated from spectrophotometric analysis of the gel placed in 2 mm cuvettes (the gel had been allowed to settle overnight in the above Tris buffer, pH 9.0) with the reference Sepharose in the blank cuvette. Alternatively, the gel was suspended in 1% polyethylene glycol (Polyox WSR 301) and the bound nucleotide analogue was determined spectrophotometrically [6]. The immobilized coenzyme analogue concentration was calculated from A_{340} using the molar absorption coefficient of unsubstituted NADH, $6.22 \times 10^3 \, M^{-1} \cdot cm^{-1}$ [9]. Irrespective of spectrophotometric method used, it was found that 15–20 nmol were bound/ml settled gel.

2.4. Fluorescence measurements

Fluorescence emission spectra of NADH, the HLADH–NADH binary complex, and the HLADH–NADH–isobutyramide ternary complex were studied. The fluorescence measurements were carried out in a Perkin-Elmer spectrophotofluorometer (MPF-2A) equipped with a thermostated cuvette holder. All samples were illuminated at 330 nm wavelength [10] and the spectra recorded from 380 nm to about 500 nm (the bandwidth of the excitation and fluorescence light was 10 nm). The fluorescence was measured in 0.1 M sodium phosphate buffer, pH 7.5 and at 25°C.

A flow-cell developed for solid phase fluorometry [4], measuring the fluorescence emitted from the surface of a packed gel bed, was used to investigate the fluorescence of the immobilized systems. About 100 µl agarose gel were packed in the flow-cell and equilibrated with the phosphate buffer. Prior to each fluorescence measurement, 4 ml sample solution were passed through the cell using a peristaltic pump but when the fluorescence spectra were recorded the pump was stopped. The gels were exposed to the excitation beam only for the time required to record the fluorescence emission spectrum and in order to diminish interference from scattered light a filter that absorbs nearly all radiation below 390 nm was selected.

Fluorescence spectra in free solution were measured with a conventional 1.0×1.0 cm fluorescence cuvette but otherwise the conditions were identical with those chosen for the immobilized systems.

2.5. Affinity chromatography

All chromatographic procedures were performed at 4°C. About 3 ml NADH–Sepharose were packed in a column and equilibrated with 0.1 M sodium phosphate buffer, pH 7.5, containing 0.1 M isobutyramide. HLADH was applied in this buffer containing the isobutyramide and was strongly adsorbed on the NADH–gel. Subsequently, the enzyme was eluted on omission of the amide from the buffer. Alcohol dehydrogenase activity was measured as in [11].

2.6. Radioactivity measurements

An aqueous sample (1 ml) was mixed with 10 ml scintillation solution containing 5.5 g PPO and 0.1 g dimethyl POPOP/litre in a 2:1 mixture of toluene and Triton X-100. Radioactivity was measured in a liquid scintillation counter (Searle, Mark III).

3. Results and discussion

The dimeric enzyme horse liver alcohol dehydrogenase is known to form a strong ($K_D = 5.5 \times 10^{-9}$ M) and highly fluorescent complex with NADH in the presence of an excess of the enzyme inhibitor isobutyramide [12]. Figure 1 shows the affinity chromatography pattern obtained when HLADH was applied to NADH–Sepharose in buffer containing 0.1 M isobutyramide. No enzyme activity could be detected until the amide was omitted from the irrigant (without any isobutyramide present in the buffer, HLADH was only retarded on the NADH–Sepharose). An identical elution profile was obtained when, instead of activity measurements, the eluted fractions were titrated with unsubstituted NADH in the presence of 0.1 M isobutyramide [10]. This method of elution, i.e. 'negative elution', has also been applied by others in affinity chromatography, for example in the purification of lactate dehydrogenase [13] and alcohol dehydrogenase [14].

Iodoacetate inhibits HLADH by selectively carboxymethylating 1 cysteine residue/subunit [15]. This has been identified as Cys-46 which is positioned in the active-site region of the subunit [16,17]. To further elucidate the nature of the binding of alcohol dehydrogenase to the immobilized NADH analogue,
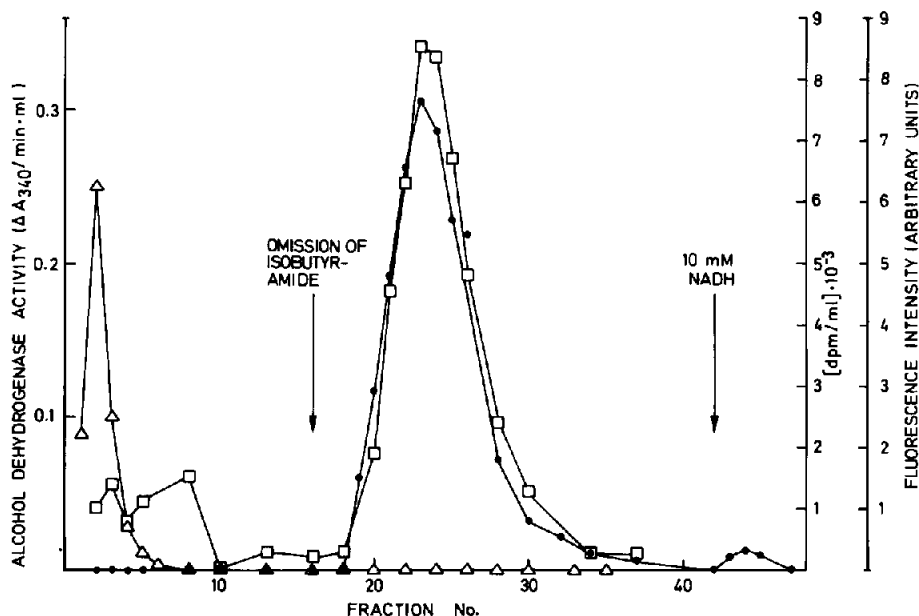
Fig.1. Affinity chromatography of HLADH on a NADH—Sepharose column (1.3 × 7 cm, containing 3 ml settled gel). About 0.5 mg HLADH in 1 ml 0.1 M sodium phosphate buffer, pH 7.5 containing 0.1 M isobutyramide was applied to the column pre-equilibrated with this buffer plus isobutyramide. Elution was effected either by omitting the isobutyramide from the irrigating buffer or by addition of soluble unsubstituted NADH to the irrigant, as indicated by the arrows. Fractions, 1.9 ml, were collected at a flow rate of 16 ml/hr. (●—●) HLADH activity; (△—△) ^{14}C-radioactivity of carboxymethylated HLADH; (□—□) fluorescence intensity of the HLADH—NADH—isobutyramide ternary complex at 410 nm (excitation wavelength 330 nm).

carboxymethylated enzyme, prepared by reacting HLADH with a 600-fold excess of radioactive iodo-acetate [15], was applied to the NADH—Sepharose. As shown in fig.1, all protein was eluted in the wash-through volume even in the presence of 0.1 M isobutyramide. Eluted protein was detected by measuring the radioactivity instead of the enzyme activity since carboxymethyl HLADH is virtually inactive and shows only about 2.5% ethanol activity of the untreated enzyme [18]. The elution pattern of active-site-modified enzyme agrees well with the suggestion made that, as a result of alkylation, ternary complexes involving HLADH and NADH plus either aldehyde or inhibitors are significantly weakened [18]. Furthermore, an increased dissociation constant for binding of NADH to alkylated enzyme has also been reported [18,19]. Thus, based on the affinity pattern obtained, it is suggested that the strong binding of the native enzyme in the presence of isobutyramide to the immobilized NADH analogue (deliberately present at very low concentrations, about 20 nmol/ml

settled gel) must be attributed to ternary complex formation on solid phase. The small amount of enzyme subsequently eluted with 10 mM of unsubstituted NADH could represent enzyme that remained bound in a binary complex.

Direct fluorometric measurements on solid phase were carried out to investigate the nature of the interactions found in the affinity studies. Thus, binary and ternary complex formation of NADH—Sepharose either with HLADH alone or together with isobutyr-amide was investigated. As is shown in fig.2a, NADH—Sepharose equilibrated with soluble alcohol dehydrogenase alone, probably forming a binary complex [12], caused a slight enhancement of the fluorescence when illuminated at 330 nm wavelength compared to the fluorescence found for the matrix-bound NADH analogue alone. On the other hand, when the same gel was equilibrated with enzyme solution but now supplemented with isobutyramide (final conc. 0.1 M), a large increase of the fluores-cence was observed which indicated the formation
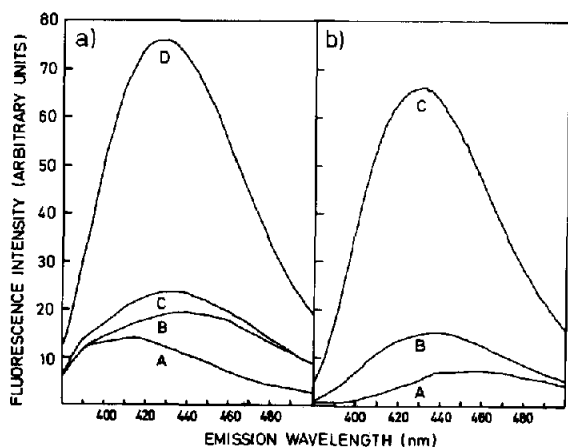
Fig.2. (a) Fluorescence emission spectra obtained with NADH–Sepharose: A, reference Sepharose equilibrated with 2.4 μN HLADH in 0.1 M sodium phosphate buffer, pH 7.5 containing 0.1 M isobutyramide; B, NADH–Sepharose equilibrated with buffer plus 0.1 M isobutyramide; C, NADH–Sepharose equilibrated with 2.4 μN HLADH in buffer; D, NADH–Sepharose equilibrated with 2.4 μN HLADH in buffer plus 0.1 M isobutyramide. (b) Fluorescence emission spectra with soluble NADH: A, 0.8 μM unsubstituted NADH; B, 0.8 μM unsubstituted NADH plus 0.4 μN HLADH; C, 0.8 μM unsubstituted NADH plus 0.4 μN HLADH and 0.1 M iso-butyramide. All spectra were measured in 0.1 M sodium phosphate buffer, pH 7.5 at 25°C. The excitation wave-length was 330 nm. The spectra illustrated in fig.2a are not on the same intensity scale as those in fig.2b.

of a ternary complex [12].

A slow release of ligands covalently bound to CNBr-activated Sepharose has been reported to take place in affinity chromatography [20] but it is highly unlikely that the emission spectra, obtained in these studies on solid phase, are due to detached non-covalently bound NADH analogues interacting with the enzyme or the enzyme plus isobutyramide since the gels had been thoroughly washed with salt and buffer immediately prior to the fluorescence measurements. In agreement with this, it was found that even after washing the NADH–Sepharose, (pre-equilibrated with enzyme plus isobutyramide), in the flow-cell with large volumes of buffer con-taining the amide (usually about 40 column vol.) the enzyme still remained affinity-bound on solid phase as ternary complex.

The flow-cell packed with reference Sepharose emitted some light in the spectral region of the NADH fluorescence. However, this emission was found to be due to interfering light emanating mostly from the flow-cell itself since CNBr-activated Sepharose alone has not been found to fluoresce [4]; a similar emission spectrum was obtained on illuminating the flow-cell containing only buffer solution. It is perti-nent to note that reference gel equilibrated with enzyme plus isobutyramide but lacking NADH did not show any significant enhancement of the fluores-cence intensity over that caused by the cell packed with only reference Sepharose.

Figure 2b shows the fluorescence spectra obtained in free solution with unsubstituted NADH alone, and as binary and ternary complexes with alcohol dehydrogenase. Apart from the effect of the blank emission on the spectral maximum of the immobilized samples resulting in a shift towards shorter wave-lengths (cf. fig.2a,b) qualitatively identical spectra were obtained for the immobilized and soluble systems. In this context it also deserves mentioning that roughly identical fluorescence spectra were found for ternary complexes in solution involving either unsubstituted NADH or the NADH analogue.

Summarizing, we feel that fluorescence measure-ments of the type described in this communication will aid in establishing the nature of the interaction found in affinity chromatography systems. Further-more, this solid-phase technique has potentially wide application in the study of interactions between other complementary pairs of molecules such as hormone and receptor or lectin and protein, provided that fluorescence measurements are possible in such systems.

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