A SIMPLE GENERAL METHOD FOR THE PREPARATION OF '6-IMMOBILIZED' ANALOGUES OF AMP, ATP, NAD AND OF OTHER ADENINE-CONTAINING COMPOUNDS FOR AFFINITY CHROMATOGRAPHY

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1. Introduction

Considerable interest has recently been shown in the preparation of immobilized 'general ligands', i.e. substances such as NAD, NADP, ATP and AMP which act as ligands or cofactors for groups of enzymes. This interest derives both from the need for such immobilized derivatives in enzyme reactors [1–3] and from the potential usefulness of such derivatives in affinity chromatography [4–12]. The usefulness of immobilized ligands in studies of enzyme binding mechanisms has also been demonstrated [10–13]. A common feature of the majority of 'general ligands' is the presence of an adenine residue. The 6 position of this residue (cf. fig. 1) would appear to be a desirable immobilization point since available specificity data suggest that the amino group in this position may often be eliminated or substituted without any drastic deterioration in the binding properties or effectiveness of the cofactors [5, 7, 10, 14].

Unfortunately this 6-amino group is unusually inert and recent studies have shown that some previous attempts at immobilization of NAD^+ through this group, by carbodiimide-promoted attachment to carboxylated polymers, resulted instead in attachment largely through the ribosyl hydroxyl groups [10]. Attachment of AMP through the 6-amino group has indeed been achieved, but only by indirect means, involving relatively complex synthetic chemical procedures and relatively extreme conditions [7]. The difficulties of such approaches seem likely to be magnified in the case of more complex and unstable ligands such as NAD^+. A recently described method making use of succinylated derivatives of NAD^+ shows some promise [3], although the specificity of the linkage procedure has not been established.

The present communication describes simple procedures for the preparation of effective 6-immobilized analogues of adenine-containing compounds. Attachment through the 6 position is achieved by using readily available, or readily preparaed, analogues in which the 6-amino group is replaced by a 6-mercapto group (fig. 1). The mercapto group condenses specifically and spontanaeously under mild conditions with bromoacetylated matrix-spacer-arm assemblies.

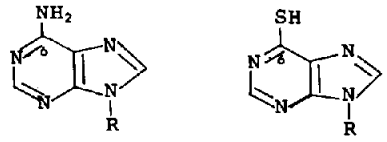

Fig. 1. Adenine derivatives (right) and their 6-mercaptopurine analogues (left).

2. Experimental

2.1. *Materials*

Sepharose 4B was obtained from Pharmacia, Uppsala. NMN, 6-mercaptopurine riboside-5'-phosphate, 6-mercaptopurine riboside and 6-methylmercaptopurine riboside were supplied by Sigma Chemical Co., London. Dicyclohexylcarbodiimide was obtained

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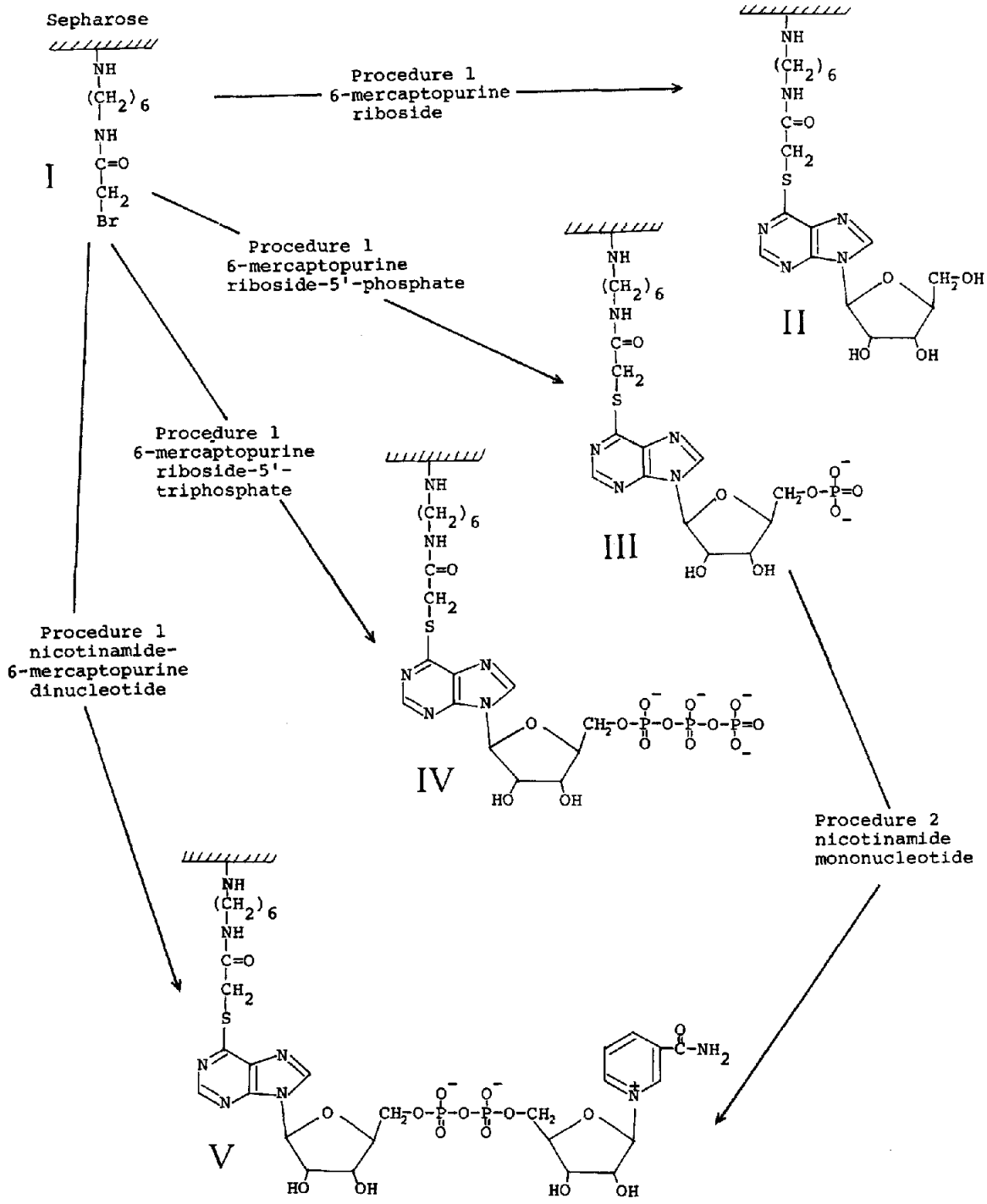

Fig. 2. Structures and preparative routes of the 6-immobilized analogues. For details of procedures 1 and 2, see Experimental section.

from BDH Chemicals Ltd., Poole. The preparation and purification of 6-mercaptopurine riboside-5′-triphosphate and nicotinamide-6-mercaptopurine dinucleotide are as described by Murphy et al. [15] and Todd et al. [16] respectively.

The enzymes were all supplied by Sigma Chemical Co., London. Lactate dehydrogenase was assayed following the method of Fritz et al. [17] but using 0.02 M phosphate buffer, pH 7.4, instead of Tris. Adenosine deaminase was assayed by the method of Kalckar [18].

2.2. *Preparation of the immobilized ligands*

The matrix-spacer-arm assembly terminating in a bromoacetyl group (I in fig. 2) was prepared from Sepharose 4B following procedures described by Cuatrecasas [19].

The synthetic procedures involved in the linkage and build-up of the ligands, as illustrated in fig. 2, are as follows:

Procedure 1

The bromoacetylated assembly (I, fig. 2, or any similar bromoacetylated analogue) is washed with 0.1 M $NaHCO_3$, pH 9.0, and the moist, packed gel is mixed with an equal volume of the same buffer containing the 6-mercaptopurine analogue at a concentration of about 15 μmoles per ml. The mixture is incubated at room temperature (15°–20°C) for five days with occasional stirring and the gel is then washed on a sintered-glass funnel with about 200 vol of 0.2 M NaCL. Any residual unreacted bromoacetyl groups are blocked by further treatment of the gel with an equal volume of 0.2 M 2-mercaptoethanol in 0.01 M $NaHCO_3$, pH 9.0, for 2 hr at 30°C. The gel is finally washed with 0.2 M NaCl (approx. 200 vol) and with distilled water (10 vol).

Procedure 2

The immobilized AMP analogue (derivative III, fig. 2) is washed on a sintered-glass funnel with 80% aqueous pyridine (v/v) and mixed with an equal volume of 80% aqueous pyridine (v/v) containing NMN (free acid) at a concentration of 10 μmoles per ml. The mixture is cooled to 0°–4°C, 93 mg (450 μmoles of dicyclohexylcarbodiimide per ml of gel is added and dissolved, and the mixture is incubated at 0°–4°C for 8 days with occasional mixing (dicyclohexyl urea begins to precipitate out after 3 to 4 days). The gel is then washed briefly on a sintered-glass funnel with about 12 vol of methanol followed by about 3 vol of 80% aqueous pyridine. The reaction procedure is repeated, using the same quantities of NMN and dicyclohexylcarbodiimide, but extending the incubation time to 16 days at 0°–4°C (the formation of dicyclohexyl urea is slower during this second incubation). The gel is washed as before and the reaction procedure is repeated once more but prolonging the incubation period to 30 days. (This final prolonged incubation is optional). The gel is finally washed with methanol (about 60 vol) followed by 0.02 M NaCl (about 100 vol) and distilled water (10 vol).

2.3. *Spectral monitoring and chromatographic procedures*

Absorbance spectra of the immobilized nucleotides were recorded using suspensions of the gels in distilled water and a Unicam S.P. 1800 spectrophotometer. The sample and reference cells were mounted near the photodetector to minimize light scattering. The blank sample in the reference compartment consisted of a suspension of the matrix-spacer-arm assembly (I, fig. 2) without any attached ligand. The contents of the cells were mixed by inversion immediately prior to recording the spectra.

The Sepharose-linked NAD^+ analogue was chemically reduced by addition of 250 mg of sodium dithionite to 1 ml of nucleotide gel suspended in 20 ml of 1.3% $NaHCO_3$. Nitrogen (99.9%, oxygen-free) was bubbled through the suspension and the mixture was sealed and incubated at 15°C for 2 hr with continuous mixing. The gel was then washed on a sintered-glass funnel with 0.2 M NaCl (100 vol) and distilled water (10 vol).

Enzymic reduction of the Sepharose-linked NAD^+ analogue was monitored as follows: the moist gel (0.3 ml) was washed with 0.03 M sodium pyrophosphate, pH 8.8, containing 0.5 M KCl and then incubated at 30°C in a spectrophotometric cuvette containing 2.4 ml of the same buffer, 0.2 ml of ethanol and 5 units of yeast alcohol dehydrogenase. The reference cell contained the same ingredients minus the alcohol dehydrogenase. The contents of the cells were kept continually mixed by inversion of the tightly capped cells.

Chromatography was carried out at 15°C, using miniature columns (7 mm internal diameter; 3 ml–4 ml bed volume) and a hydrostatic head of pressure

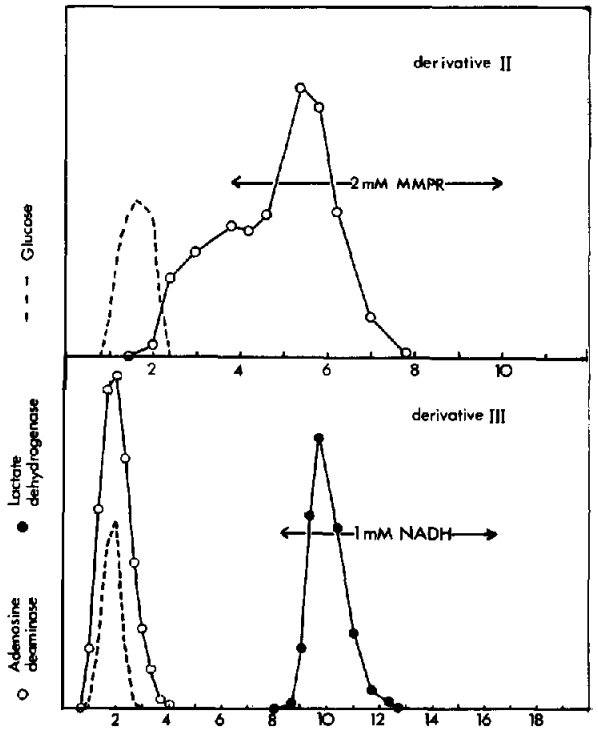

Fig. 3. Affinity chromatography of adenosine deaminase on the 6-immobilized adenosine analogue (derivative II) and of lactate dehydrogenase on the 6-immobilized AMP analogue (derivative III). The irrigant for both affinity columns was 0.1 M KCl in 0.1 M potassium phosphate buffer, pH 7.0, with the addition of the appropriate counter ligand to the irrigant indicated by the horizontal arrows. Effluent volume was measured from the time of application of the enzyme samples. The breakthrough position is marked by the elution peak of a trace of glucose, applied with each enzyme sample and monitored in the effluent as previously described [13]. For further details see Experimental section. (MMPR: methylmercaptopurine riboside; (o—o—o) adenosine deaminase; (•—•—•) lactate dehydrogenase; (– – –) glucose.

adjusted to give a flow rate of one column volume per 10 min. Enzyme samples of 0.4 ml were applied and the effluent was collected as 0.4 ml fractions using a Gilson microfractionator. The effluent volume is expressed in terms of column volume units (fig. 3), calibration being carried out as previously described [13].

3. Results and discussion

Procedure 1 (fig. 2) depends on the spontaneous condensation of the thiol group of the mercaptopurine residue with the reactive bromoacetyl group, HBr being eliminated. This procedure is both simple and specific and it has the advantage of involving only mild conditions. The resulting immobilized 6-mercaptopurine derivatives have an absorbance spectrum (λ_{max} 290 nm) very similar to those of S-substituted 6-mercaptopurine derivatives generally, and differing from those of 6-mercaptopurine derivatives in which the mercapto group remains unsubstituted. For example under the same conditions 6-methylmercaptopurine riboside also has its absorption maximum at 290 nm while 6-mercaptopurine riboside and its phosphorylated derivatives have the absorbance maximum at 322 nm [20]. The spectral difference is due to tautomerization of the free mercapto group which is blocked in the S-substituted derivatives. This is a clear indication that the immobilization linkage is through the 6-mercapto group as expected.

The chemistry of procedure 2 is not as clear-cut or precise as that of procedure 1. It is based on the synthetic method used originally by Todd et al. [16] for the synthesis of NAD^+ by carbodiimide-promoted condensation of AMP and NMN in free solution. (This same method is also used for the synthesis of the 6-mercaptopurine analogue of NAD in free solution [15]. Self condensation of AMP and of NMN are side-reactions complicating the free-solution reaction and necessitating separation of the NAD^+ product from the side products. However, such complications were not expected to be troublesome in the condensation of NMN with the immobilized AMP analogue (fig. 2), since the immobilized state of the AMP analogue seems to preclude self-condensation, while the product of self-condensation of NMN is simply removed in the wash.

The immobilized analogue resulting from procedure 2 developed a second absorbance peak at 340–342 nm upon reduction with sodium dithionite, confirming that NMN had been incorporated. The intensity of this absorbance was consistent with 1:1 incorporation of NMN relative to the immobilized 6-mercaptopurine analogue. This initially suggested quantitative and specific formation of the immobi-

lized NAD^+ analogue (derivative V, fig. 2) by procedure 2. However, by the same spectrophotometric criterion only about 20% reduction was achievable enzymically when this immobilized analogue was incubated in the presence of alcohol dehydrogenase and ethanol. Under the same conditions alcohol dehydrogenase fails to reduce NMN to a detectable extent, so that it can be assumed that at least 20% of the NMN incorporated by procedure 2 is 'correctly' linked, to the AMP analogue to form an enzymically effective NAD^+ analogue. The reason for the ineffectiveness of the remaining 80% is unclear. It may reflect a lack of specificity in procedure 2 resulting in a high proportion of 'incorrectly' linked ligand molecules, or it may reflect inhomogeneity in the agarose matrix with dense regions inaccessible to the enzyme.

The proportion of 'effective' NAD^+ analogue residues in derivative V is sufficient to provide an effective affinity gel, as mentioned below. Direct linkage of the complete, pre-assembled mercaptopurine analogue of NAD^+ to the bromoacetylated gel (indicated as an alternative preparative route in fig. 2) clearly has the advantage of more reliably yielding a more homogeneous population of effective immobilized ligand residues. However, the cost and trouble of the preassembly steps have thus far made the easier and less expensive modular solid-phase assembly approach a much more attractive proposition, although the product is admittedly less well-defined chemically.

Efforts to apply a solid-phase modular approach, similar to procedure 2, in the synthesis of the ATP analogue (derivative IV, fig. 2) by carbodiimide-promoted attachment of a pyrophosphate group to derivative III were much less successful. In this case the free-solution synthesis of the ATP analogue is relatively simple and free of complications so that the preassembly approach as indicated in fig. 2 seems preferable in this case.

The immobilized adenosine analogue (derivative II, fig. 2) was an effective affinity gel for adenosine deaminase, although the affinity was relatively weak, as indicated by the slow leakage of the enzyme from the columns (fig. 3). The enzyme could be specifically and cleanly eluted from the column by addition of 6-methylmercaptopurine riboside to the irrigant as a counter ligand (fig. 3), confirming the biospecificity of the adsorption. This was further indicated by the lack of affinity of the corresponding AMP-analogue gel (derivative III, fig. 2) for adenosine deaminase (fig. 3), in agreement with the known specificity of this enzyme.

By contrast, this 'AMP' gel adsorbed lactate dehydrogenase biospecifically, the specificity of binding being shown by the specific elution of the enzyme from this gel by addition of NADH as a counter ligand to the irrigant (fig. 3). (AMP represents half the NAD^+ molecule and is an inhibitor of lactate dehydrogenase competitive with respect to NAD^+ and NADH). Derivative III showed similar biospecific affinity for some other AMP-binding enzymes, such as liver alcohol dehydrogenase, but it promoted only marginal adsorption of others, such as malate dehydrogenase, and it showed no detectable affinity at all for adenylate kinase (myokinase from rabbit muscle). Some other AMP-specific enzymes, such as AMP deaminase from rabbit muscle, were very strongly adsorbed in an essentially irreversible fashion, seemingly by non-biospecific interactions with the hydrocarbon spacer-arm. Such non-biospecific adsoption completely eclipsed any biospecific affinity for the ligand (T. Griffin and P. O'Carra, unpublished results).

Thus the general effectiveness envisaged in the original concept of general-ligand affinity chromatography [4, 5] is considerably attenuated in the case of this immobilized AMP analogue. The advantages as well as the disadvantages of such attenuation have been discussed elsewhere [10,21] in relation to somewhat similar behaviour which we have noted in the case of certain other immobilized 'general-ligands' – in particular certain immobilized NAD^+ derivatives attached via an azo-linkage, apparently through the 8 position of the adenine residue [10].

By contrast with the patchy effectiveness of these azo-linked NAD^+ derivatives the 6-linked NAD^+ analogue described here (derivative V, fig. 2), was a very effective affinity adsorbent for all the NAD^+ linked dehydrogenases so far tested. These include lactate, malate, alcohol and glyceraldehyde-3-phosphate dehydrogenase. Thus derivative V comes much closer to the general-ligand ideal, but it remains to be established whether this is an unqualified advantage (cf. refs. [10, 21]).

A comprehensive study of the affinity chromatographic applications of these immobilized derivatives and of the ATP analogue (derivative IV), both in enzyme purification and in mechanistic studies, is in progress and will be published elsewhere.

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