The Immobilization of Adenine Nucleotides on Polysaccharides by using Glutaraldehyde Coupling and Borohydride Reduction

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Adenine nucleotides were immobilized on modified Sepharose 4B or Dextran T40 with glutaraldehyde and reduced with KBH_4 . Binding was dependent on pH and the nature of the amino group on the modified polysaccharide. ATP bound to soluble dextran retained coenzyme activity with glycerol kinase. Binding is proposed to occur via a Schiff base.

Numerous methods have been developed for the immobilization of nucleotides on a variety of inert supports, although emphasis has been placed on the coupling of adenine-containing nucleotides (see Lowe & Dean, 1974, for a review). Several different points of attachment are available on adenine nucleotides, but the N-6 position is preferable, as it provides suitable affinity columns (Lowe *et al.*, 1973) and partial retention of coenzyme activity (Lindberg *et al.*, 1973). These methods have proved suitable for the more stable compounds such as NAD and AMP, but involve reaction conditions that would be too harsh for the coupling of labile nucleotides such as ATP. Where procedures have been developed for ATP, yields are often low and the chemistry is complicated (Eckstein *et al.*, 1975).

Use of a bifunctional reagent such as glutaraldehyde has the advantage of simplicity and mild reaction conditions, and it could be used to couple ATP to an amino derivative of a polysaccharide. When Mansson *et al.* (1976) coupled acriflavins to an amino derivative of Sepharose using glutaraldehyde, the linkage was found to be unstable, but this problem could be overcome by reduction with KBH_4 . A dextran-ATP complex made by this method was found to be completely stable (Gacesa & Whish, 1977).

It was decided to investigate the mode of attachment of adenine nucleotides on amino derivatives of polysaccharides by using glutaraldehyde coupling and borohydride reduction.

Materials and Methods

Sephadex G-25 (fine grade), Dextran T40 and Sepharose 4B were purchased from Pharmacia, London W.5, U.K. $[2\text{-}^3\text{H}]\text{Adenosine}$, $[2\text{-}^3\text{H}]\text{ATP}$, $[\text{U}\text{-}^{14}\text{C}]\text{inosine}$ and $[\text{methyl}\text{-}^3\text{H}]\text{thymidine}$ were obtained from The Radiochemical Centre, Amersham,

Bucks., U.K. Glycerol kinase (EC 2.7.1.30), pyruvate kinase (EC 2.7.1.40), lactate dehydrogenase (EC 1.1.1.27), phosphoenolpyruvate and nucleotides were purchased from Boehringer Corp. (London) Ltd., Lewes, Sussex, U.K. CNBr, 1,2-diaminoethane and 1,4-diaminobenzene were obtained from Aldrich Chemical Co., Gillingham, Dorset, U.K. All other chemicals were purchased from BDH, Poole, Dorset, U.K., and were of AnalaR grade or the highest purity available.

Sepharose 4B was activated with CNBr, a derivative with 1,4-diaminobenzene prepared (Porath & Axen, 1976) and stored at 4°C until required in aq. 50% (v/v) ethanol containing 10mM-mercaptoethanol. To couple nucleotides to the amino derivative of Sepharose, 1.2ml of packed, damp gel was thoroughly washed on a Buchner funnel with 300ml of water, dried by suction and placed in a graduated glass centrifuge tube. The gel was resuspended in 2ml of water and 3ml of aq. 25% (w/v) glutaraldehyde. This mixture was allowed to react for 30min at room temperature (22°C), then filtered and washed with water to remove excess glutaraldehyde. The gel was resuspended in 15ml of 50mM-sodium citrate buffer, pH 6.0, containing 0.24mmol of $[2\text{-}^3\text{H}]\text{adenosine}$ and the reaction allowed to proceed for 30min at room temperature. Other nucleotides were treated similarly. In certain experiments, as indicated in the Results section, citrate buffers of different pH values were used in the nucleotide-coupling stage. Subsequent to coupling the solution was adjusted to approx. pH 9 with 2M-NaOH, and 0.2g of solid KBH_4 added. After 15min reduction the gel was filtered and washed extensively with 1M-NaCl and water to remove any non-covalently bound material.

A derivative of Dextran T40 with 1,4-diaminobenzene was prepared and coupled with ATP as described previously (Gacesa & Whish, 1977). The 1,2-diaminoethane derivative of Dextran T40 was prepared by the same method. Bound and free ATP were separated on a column (0.5cm×46.0cm) of

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Sephadex G-25 (fine grade) in 25 mM-Tris/HCl buffer, pH 7.5, either with or without 1 M-NaCl, at a flow rate of 7.5 ml/h. Bound nucleotides were measured by liquid-scintillation counting [0.5% (w/v) 2,5-diphenyloxazole in Triton/toluene (3:7, v/v)], by total phosphorus (Bartlett, 1959) or by u.v. absorbance (by assuming $\epsilon = 15700 \text{ litre} \cdot \text{mol}^{-1} \text{ cm}^{-1}$).

In all kinetic experiments the initial reaction rates of glycerol kinase were followed by using phosphoenolpyruvate, pyruvate kinase and lactate dehydrogenase to couple the rates of ADP formation to the oxidation of NADH. Decrease in A_{340} was measured at 25°C. Reaction mixtures (final pH 7.5) contained 25 mM-Tris/HCl buffer, pH 7.5, 1 M-NaCl, 0.13 mM-NADH, 3.04 mM-phosphoenolpyruvate, 2.6 mM-MgCl₂, 50 μg of pyruvate kinase, 100 μg of lactate dehydrogenase, 25 μg of glycerol kinase and various concentrations of substrates in a total volume of 1.000 ml. Typically, reactions were started by the addition of glycerol kinase. Control experiments were carried out to ensure that the reaction catalysed by glycerol kinase was rate-limiting, and that the 1,4-diaminobenzene derivative of Dextran T40 did not alter reaction rates with free ATP as substrate. The activity of glycerol kinase was monitored before and after each set of experiments. Kinetic constants were obtained by using direct linear plots (Eisenthal & Cornish-Bowden, 1974).

Results

Adenosine and ATP were found to bind covalently to the 1,4-diaminobenzene derivative of Sepharose 4B, but neither inosine or thymidine bound significantly (Table 1). The pH of the reaction mixture was critical. When ATP was allowed to react with the Sepharose-glutaraldehyde complex at low pH values an increased amount of ATP was bound (Fig. 1).

ATP was coupled to a 1,4-diaminobenzene derivative of a soluble dextran in good yields, as estimated by total phosphorus (26 μmol of ATP/g dry wt. of dextran, i.e. 78 μmol of phosphorus/g) after separation of the remaining free ATP on

Table 1. Covalent binding of nucleotides to Sepharose 4B

The binding of nucleotides to the 1,4-diaminobenzene derivative of Sepharose 4B was quantified by liquid-scintillation counting by using [2-³H]adenosine (56 Ci/mol), [2-³H]ATP (18 Ci/mol), [methyl-³H]-thymidine (25 Ci/mol) and [U-¹⁴C]inosine (0.35 Ci/mol). See the text for details.

Nucleotide bound		
Nucleotide	($\mu\text{mol}/\text{ml}$ of Sepharose)	Relative binding
Adenosine	2.13	100
ATP	1.20	56
Inosine	0.05	2
Thymidine	0.02	1

Sephadex G-25 in the presence of 1 M-NaCl. Contrasting results were obtained with the 1,2-diaminoethane derivative of dextran. When this derivative was treated with ATP and chromatographed on Sephadex G-25 in the absence of 1 M-NaCl all of the ATP was eluted in the void volume. In the presence of 1 M-NaCl, however, the complex dissociated and ATP was eluted as a different peak considerably after the void volume (Fig. 2). An identical pattern was obtained if free ATP was added to the 1,2-diaminoethane derivative of dextran in the absence of glutaraldehyde and chromatographed. ATP coupled to the 1,4-diaminobenzene-dextran was stable over 4 months when stored at -30°C. Rechromatography of the sample after this time showed no sign of free ATP when eluted with 1 M-NaCl.

ATP coupled to dextran by the method reported here acted as a substrate for glycerol kinase. A preliminary kinetic analysis of glycerol kinase indicated that at high concentration of glycerol (500 mM), the apparent K_m for free ATP was found to be 140 μM , compared with 20 μM for the coupled ATP. The relative maximum velocity was decreased from 1.0 for free ATP to 0.12 for the immobilized ATP.

Discussion

The results in Table 1 show that glutaraldehyde may be used successfully to couple certain nucleotides to amino derivatives of polysaccharides. Both ATP and adenosine bound to 1,4-diaminobenzene derivatives of Sepharose, via glutaraldehyde, in yields that agree well with those obtained by other methods (Craven *et al.*, 1974; Eckstein *et al.*, 1975; Mansson

Fig. 1. Dependence of ATP immobilization on pH [2-³H]ATP (18 Ci/mol) was allowed to react with the 1,4-diaminobenzene derivative of Sepharose 4B in a range of citrate buffers and binding was estimated by liquid-scintillation counting. See the text for details.

Fig. 2. Elution of ATP-1,2-diaminoethane-dextran complex on Sephadex G-25

The 1,2-diaminoethane derivative of dextran (12.5 mg), complexed with ATP, was chromatographed on a column (0.5 cm $\times$ 46.0 cm) of Sephadex G-25 (fine grade) in 25 mM-Tris/HCl buffer, pH 7.5, with (Δ) and without ($\triangle$) 1M-NaCl: 0.5 ml fractions were collected at a flow rate of 7.5 ml/h. V_0 indicates the void volume.

et al., 1976). However, the method reported here has the real advantage of simple chemistry and gentle reaction conditions, thus facilitating the coupling of the more labile coenzymes such as ATP. Another advantage is that reaction times are considerably shorter with this method; coupling typically takes about 1 h.

Neither inosine nor thymidine bound in significant amounts compared with the adenine nucleotides. This indicates that the primary amino group of adenine is the position through which the linkage is made, as there are no other groups on the nucleotides that are likely to react with an aldehyde under the conditions used. Further evidence is provided by the shift in the u.v.-absorbance maximum from 259 to 266 nm that was observed when ATP was coupled to dextran by this method (Gacesa & Whish, 1977). It is proposed that coupling of the amino group on either the Sepharose derivative or the nucleotide with the aldehyde groups of glutaraldehyde occurs via a condensation reaction to produce a Schiff base. Reactions of this type are considered to be acid-catalysed (Roberts & Casserio, 1965) and it would be expected that the binding of nucleotide would increase as the pH of the reaction mixture is lowered. Experimentally this was found to be the case (Fig. 1).

Mechanistically, the initial step of the reaction (Scheme 1) is thought to be a nucleophilic attack by the unprotonated amino group, $R-NH_2$, on the carbonyl group to form an adduct (I) (Sykes, 1968). If the pH is too low then an insufficient proportion of

Scheme 1. Proposed mechanism of Schiff-base formation

The nucleophilic attack of the unprotonated amino group on the carbonyl group to form the adduct (I) is rapid and followed by a rate-limiting acid-catalysed dehydration to yield the Schiff base.

the amino groups will be unprotonated and the formation of adduct (I) will be slow. Evidently the pK value of the free amino group will be instrumental in controlling reaction rates. By using this mechanism it is possible to explain the lack of covalent binding when the dextran was coupled with 1,2-diaminoethane. The pK of the amino group on the 1,2-diaminoethane derivatives of dextran will be approx. 10.5, compared with a value of about 4.5 for the 1,4-diaminobenzene derivative of dextran. Therefore only negligible amounts of the amino group on the 1,2-diaminoethane derivative would be unprotonated when the coupling procedure is followed at pH 6. The observed non-covalent binding of ATP with the 1,2-diaminoethane derivative of dextran (Fig. 2) is probably an ionic interaction between the positive charge on the amino group and the negative charges on ATP.

Dextran-ATP appears to be a satisfactory substrate for glycerol kinase. It is surprising to find the $K_{m(\text{app.})}$ for ATP lowered by a factor of 7 after derivative formation, although similar results were obtained by Fuller & Bright (1977) when they coupled ATP to an acrylic co-polymer and used it as a substrate for hexokinase. This is in contrast with the results obtained by Weibel *et al.* (1974), where the $K_{m(\text{app.})}$ for NAD^+ was considerably raised on immobilization to dextran.

The reported instability of glutaraldehyde coupling has not been observed. Reduction of the Schiff bases with borohydride in this method would prevent the possibility of hydrolysis that has been observed for unreduced compounds (Mansson *et al.*, 1976). This chemistry is analogous to the isolation of substrate-enzyme complexes by reduction of the Schiff-base intermediate with borohydride before hydrolysis (Grazi *et al.*, 1962).

In conclusion, this method is suitable for the coupling of adenine nucleotides via the primary amino group to 1,4-diaminobenzene derivatives of

polysaccharides. The results in the present paper show that the method has the advantages of simplicity, speed, good yields and retention of coenzyme activity in the case of the water-soluble derivatives. The method could be applied to the immobilization of other compounds containing primary amino groups and the development of affinity-chromatography media.

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References

- Bartlett, G. R. (1959) *J. Biol. Chem.* **234**, 466–468
- Craven, D. B., Harvey, M. J., Lowe, C. R. & Dean, P. D. G. (1974) *Eur. J. Biochem.* **41**, 329–333
- Eckstein, F., Gourmet, M. & Wetzel, R. (1975) *Nucleic Acids Res.* **2**, 1771–1775
- Eisinger, R. & Cornish-Bowden, A. (1974) *Biochem. J.* **139**, 715–720
- Fuller, C. W. & Bright, H. J. (1977) *J. Biol. Chem.* **252**, 6631–6639
- Gacesa, P. & Whish, W. J. (1977) *Biochem. Soc. Trans.* **5**, 1407–1408
- Grazi, E., Rowley, P. T., Cheng, T., Tchola, O. & Horecker, B. L. (1962) *Biochem. Biophys. Res. Commun.* **9**, 38–43
- Lindberg, M., Larsson, P.-O. & Mosbach, K. (1973) *Eur. J. Biochem.* **40**, 187–193
- Lowe, C. R. & Dean, P. D. G. (1974) *Affinity Chromatography*, John Wiley, London
- Lowe, C. R., Harvey, M. J., Craven, D. B., Kerfoot, M. A., Hollows, M. E. & Dean, P. D. G. (1973) *Biochem. J.* **133**, 507–513
- Mansson, M. O., Matiasson, B., Gestrelus, S. & Mosbach, K. (1976) *Biotechnol. Bioeng.* **18**, 1145–1159
- Porath, J. & Axen, R. (1976) *Methods Enzymol.* **44**, 19–45
- Roberts, J. D. & Casserio, M. C. (1965) *Basic Principles of Organic Chemistry*, pp. 449–453, W. A. Benjamin, New York and Amsterdam
- Sykes, P. (1968) *A Guidebook to Mechanism in Organic Chemistry*, 2nd edn., pp. 164–165, Longmans Green, London
- Weibel, M. K., Fuller, C. W., Stadel, J. M., Buckmann, A. F. E. P., Doyle, T. & Bright, H. J. (1974) in *Enzyme Engineering* (Pye, E. K. & Wingard, L. B., Jr., eds.), vol. 2, pp. 203–208, Plenum, New York