IMMOBILIZATION OF GLYCOGEN PHOSPHORYLASE *b* IN ITS ACTIVE CONFORMATION ON MATRICES SUBSTITUTED WITH AN AMP-ANALOGUE

Klaus MOSBACH and Stina GESTRELIUS
Biochemical Division, Chemical Centre, University of Lund, P.O. Box 740, S-220 07 Lund 7, Sweden

Received 18 March 1974

1. Introduction

Immobilized general ligands such as analogues of NAD^+ [1] or AMP [2], have proven most valuable in biospecific affinity chromatography for the separation and purification of various enzymes [3,4] and isozymes [5]. In the present study we have extended the use of an AMP-analogue, N^6-(6-aminohexyl)adenosine 5′-monophosphate, to achieve a situation of 'permanent' activation of a regulatory enzyme. The enzyme chosen was rabbit muscle glycogen phosphorylase *b* which has an absolute requirement for the effector AMP [6–8]. Three different lines of approach were followed to this end: a) the enzyme was strongly adsorbed to Sepharose-bound AMP-analogue, b) the enzyme and the AMP-analogue were immobilized concurrently to Sepharose or alkylamine glass, and c) the enzyme was immobilized to Sepharose or alkylamine glass in the presence of AMP, to investigate whether immobilization in the presence of effector, would keep the enzyme in an active conformation even after subsequent removal of AMP by exhaustive washing. Both procedures a) and b) resulted in 'permanently' activated enzyme preparations.

2. Experimental

Glycogen phosphorylase *b* from rabbit muscle, α-D-glucose-1-phosphate, β-glycerophosphate and AMP were purchased from Sigma (St. Louis, Mo., USA); AMP-free glycogen (type I) from Boehringer (Mannheim, Germany); *n*-hexylamine and L-cysteine hydrochloride from Merck (Darmstadt, Germany) and extra pure ethylene glycol as well as 2-mercaptoethanol from BDH (Poole, England). Sepharose 4B and Dextran T40 were obtained from Pharmacia (Uppsala, Sweden) and alkylamine glass GAO 3940 from Corning (Medfield, Mass., USA). The AMP-analogue, N^6-(6-aminohexyl)adenosine-5′-monophosphate, was prepared as described elsewhere [2].

Phosphorylase *b* was bound to Sepharose 4B by the CNBr method [9] and coupling allowed to proceed overnight in a rotating test tube at 4°C in 4 ml 0.1 M $NaHCO_3$. About 1.2 mg protein and, where appropriate, 15 mg AMP or AMP-analogue were added to 2 g wet gel (0.1 g dry weight). The preparation was thoroughly washed with 0.1 M $NaHCO_3$, 0.5 M NaCl, 45% ethylene glycol in 0.1 M KCl and 0.05 M glycerophosphate–0.04 M cysteine buffer pH 6.8. Phosphorylase *b* was coupled to alkylamine glass (pore diameter 550 Å) following activation by 2.5% glutaraldehyde [10]. In general 2–4 mg protein and 20 mg AMP or AMP-analogue were used per 0.5 g dry glass in 5 ml 0.1 M glycerophosphate buffer pH 6.8. The preparations were washed as above. Coupling yields of protein were determined by amino acid analysis [11] and the amount of AMP-analogue was determined by phosphate analysis [12].

The AMP-analogue was immobilized on its own to Sepharose or to soluble dextran (MW 40 000) by the CNBr technique. The dextron preparation was purified by ethanolic precipitation and separation on a Sephadex G-50 column in 0.25 M LiCl [13]. The concentration of bound AMP-analogue was deter-

North-Holland Publishing Company – Amsterdam

mined from its absorbance at 267 nm (ϵ = 17 300) and the amount of dextran with the anthrone reagent [14].

Enzyme activities were measured in the direction of glycogen synthesis [15]. The free enzyme was passed through a Sephadex G-25 column prior to use to remove any AMP present. The immobilized preparations were assayed at 28°C in a total volume of 4.8 ml with 1% glycogen, 0–4 mM AMP or AMP-analogue and 32 mM glucose-1-phosphate, all adjusted to pH 6.8, in 19 mM glycerophosphate–15 mM cysteine buffer pH 6.8. The reaction vessel was placed in a shaker in a thermostated bath (Braun–Melsungen) and aliquots of 50 µl were withdrawn through a filter every five or ten minutes analysed for free phosphate.

In the column assays about 0.5 ml Sepharose preparation was packed in a small column and the substrate solution (1% glycogen and 32 mM glucose-1-phosphate in 50 mM glycerophosphate–15 mM mercaptoethanol buffer pH 6.8), total volume 2.0 ml, was continuously circulated at room temperature with a LKB-Beckman peristaltic pump.

3. Results and discussion

In the initial phase of this investigation the free AMP-analogue was tested to establish whether, and to what degree, it would replace AMP as an effector for the enzyme. Fig. 1 shows that phosphorylase was activated about 80% relative to unmodified AMP with a K_a, taken as the nucleotide concentration at half maximum activation, of $5 \cdot 10^{-4}$ M compared to a K_a of $8 \cdot 10^{-5}$ M for AMP. This activation was not unexpected in view of the large number of other analogues reported to affect phosphorylase *b* [16,17].

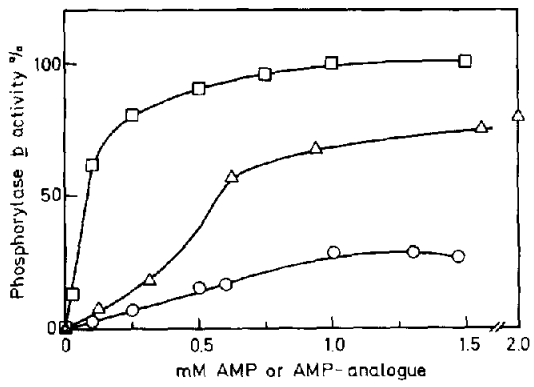

Fig. 1. Activation of soluble glycogen phosphorylase *b* with AMP (□), AMP-analogue (△) and dextran-bound AMP-analogue (○).

In the next step the analogue was immobilized through its amino-group to a water-soluble dextran to establish whether the amino-group, either on the AMP or on the analogue, is required for enzyme activation and whether a bulky support will decisively impede activation. Fig. 1 shows that these preparations (about 150 µmoles of analogue bound per g dry dextran) activated phosphorylase *b* by about 30% relative to free AMP indicating steric hindrance by the water-soluble support. Such polymer preparations may well find practical application when co-entrapped with the enzyme in polymer beads.

In the following stage attempts were made to decide whether phosphorylase could be activated by a preparation comprising the AMP-analogue immobilized to a particulate support such as Sepharose. In the test the bound effector preparation (150 µmoles of analogue per g dry Sepharose) was packed in a column as described in the experimental section and a solution containing enzyme, glucose-1-phosphate and the primer glycogen was circulated continuously through the bed. Fig. 2 shows that line A, which represents the synthesis of polysaccharide measured as the release of inorganic phosphate, was linear through the first 30 min. The amount of product formed per minute and per mg of enzyme during this initial phase was about 20% relative to that for the same amount of enzyme when activated with free AMP, or 25% compared to activation with free AMP-analogue. It is most likely that steric hindrance imposed by the matrix accounts for the lower degree of activation. In a control experiment under identical conditions but omitting the enzyme no phosphate was liberated. Furthermore, it was shown conclusively that no AMP-analogue had leaked out into the solution since addition of free phosphorylase to an aliquot of this solution did not release any free phosphate.

In a subsequent experiment, the question as to whether the enzyme is firmly bound to the immobilized effector, or is in equilibrium with the mobile

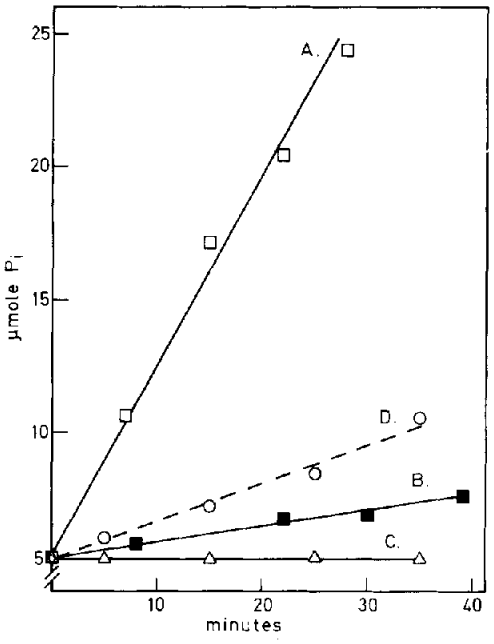

Fig. 2. Activity measurements of phosphorylase *b* in a 2 ml recycling system (15 ml/hr) over a column (7 × 0.6 cm) containing the packed Sepharose preparation. Aliquots of 50 µl were withdrawn and analysed on the contents of inorganic phosphate. A: 0.5 ml AMP-analogue-Sepharose with 0.2 mg phosphorylase *b* recycled in substrate solution (1% glycogen and 32 mM glucose-1-phosphate in 50 mM glycerophosphate–15 mM mercaptoethanol buffer pH 6.8). B: 0.5 ml AMP-analogue-Sepharose with adsorbed phosphorylase *b* (i.e. no free enzyme circulating in the solution). Recycling of substrate solution as above. C: The above Sepharose preparation after washing the column with ice-cold 45% ethylene glycol in 0.1 M KCl. D: 0.75 ml Sepharose with AMP-analogue and phosphorylase *b* both covalently bound to the matrix. This preparation had previously been washed with ethylene glycol as above.

phase, was investigated. The column was washed with buffer and then excessively with substrate solution (glucose-1-phosphate and glycogen in buffer), which eluted more enzyme. When no more enzyme was released from the column the system was recirculated as above and the activity given as line B in fig. 2 was measured. This rate is far lower than that observed in the first case (line A), where probably more enzyme is oriented around the effector and the excess of enzyme present in the circulating system is likely to displace the equilibrium even further towards formation of the enzyme-immobilized effector complex (K_{diss} for enzyme-AMP of about $1 \cdot 10^{-4}$ M has been reported in [7]). Controls revealed that no enzyme leaked from the column since the same rate was obtained at several 1 hr intervals, and no free enzyme could be detected in the solution. Thus the enzyme remained immobilized in its active conformation on the column. It is not unlikely that this binding of the enzyme includes a hydrophobic contribution from the interaction between the lipophilic hexamethylene spacer of the ligand and the hydrophobic site known to exist in the enzyme [18].

In the two other approaches to obtain 'freezing' of phosphorylase *b* in its active state, the enzyme was bound to a CNBr-activated Sepharose matrix. As is seen from Table 1, the specific activities obtained for the various preparations are relatively high and lie in the range of 15–30% compared to free enzyme. It appears that the presence of effector or effector-analogue during coupling protects the enzyme in analogy to earlier observations made during the entrapment of hexokinase in polyacrylamide beads [19]. The specific activities of Sepharose-bound phosphorylase are equivalent to those found by other authors [20].

Comparable results were obtained when the enzyme was bound to glutaraldehyde treated alkylamine glass, although the specific activities were somewhat lower, approximately 15% (2.2 µmoles P_i. $mg^{-1} \cdot min^{-1}$), with binding yields of about 50%. Whether both subunits of the dimeric enzyme (MW ~ 200 000) are covalently bound to the matrices has not yet been established, but it appears likely in view of the size of the protein and extent of activation of the matrices.

When the enzyme was coupled in the presence of 10 mM AMP no activity was found in the exhaustively washed preparation unless AMP was re-added. Evidently, the presence of effector did not assist in the stabilization of the enzyme in an active conformation that could be immobilized through the, likely occurring, multiple points of attachment. This would be in accordance with the reported concept that the effector-induced conformational changes are restricted to a small region on the enzyme [8].

However, when the enzyme was bound in the presence of the AMP-analogue (10 mM), with its free amino-group suitable for binding to a matrix, then

Table 1
Binding yield and specific activity of Sepharose-bound glycogen phosphorylase *b*.

Phosphorylase *b* preparations	Specific activity			Binding	Yield
	−AMP	+AMP (optimal)[a]	+AMP-analogue (optimal)[a]	mg protein/g dried support	% Of added protein bound
		(μmole $P_i \cdot mg^{-1} \cdot min^{-1}$)			
Soluble native phosphorylase *b*	0	14.6 (100%)[b]	12.4 (100%)[b]	–	–
Sepharose-bound phosphorylase *b*	0	2.4 (16%)[b]	2.3 (19%)[b]	5.9	48
Phosphorylase *b* bound to Sepharose in presence of 10 mM AMP	0	3.9 (27%)[b]	3.8 (31%)[b]	5.4	43
Phosphorylase *b* and AMP-analogue (10 mM) concurrently bound to Sepharose	1.4	4.3 (30%)[b]	3.8 (31%)[b]	4.2[c]	33

[a] Optimal nucleotide concentrations were in the range of 1–4 mM.
[b] The values in brackets give the specific activities of the various immobilized preparations in per cent relative to the soluble phosphorylase *b*.
[c] The amount of covalently bound AMP-analogue was 70 μmoles (30 mg)/g dried support.

active preparations were obtained even though excessive washing was carried out to remove non-covalently bound analogue. The time course of a typical preparation during recycling in a column is plotted in fig. 2 as line D. Table 1 shows that about 30% (the mean of several determinations) of the maximum activity, obtainable with a saturating concentration of native AMP, was achieved without any 'external' addition of effector. This figure corresponds to 40% of that obtainable with saturating concentrations of free AMP-analogue.

The preparations were washed with 45% ethylene glycol in 0.1 M KCl to remove any analogue bound to the enzyme by possible hydrophobic interaction. In controls run such conditions were sufficient to release the free AMP-analogue from Sepharose-bound phosphorylase. Likewise when 0.1 M AMP in 25% ethylene glycol was employed as an alternative washing procedure the free AMP-analogue was washed off, but the analogue when bound together with the enzyme to activated Sepharose, was not.

When the ethylene glycol treatment was applied to the biospecifically adsorbed enzyme preparation (fig. 2, line B) then all the enzyme was removed (fig. 2, line C). This again shows that the enzyme in the above preparations was covalently bound to the matrix, and not merely physically adsorbed to the immobilized effector.

When a glass matrix was used instead of Sepharose, similarly active phosphorylase preparations were obtained prior to any addition of soluble effector.

While this work was in progress a report appeared on the preparation of partially active phosphorylase *b* with a nucleotide-analogue covalently bound to the protein itself [21]. The enzyme precipitated, however, on increasing the nucleotide concentration, probably due to side reactions of the nucleotide with the enzyme.

To our knowledge the present study is the first report on the successful immobilization of an effector-dependent enzyme in its active conformation on a matrix such that no requirement was shown for the addition of effector to induce activity. In the case where the enzyme was simply added to the matrix-bound AMP-analogue, the high affinity of the enzyme for the immobilized effector sufficed to keep the enzyme immobilized in its active conformation.

In the alternative case the enzyme is covalently

bound to the matrix and apparently kept oriented around the immobilized effector. With these preparations the initial activities (without soluble effector added) varied and the value given in table 1 is the mean of several preparations. The competition during binding of the nucleotide and enzyme for the activated matrix may in part account for these variations. The fact that addition of free AMP or analogue does increase the degree of activation is probably due to the fact that many molecules are not oriented optimally towards the immobilized effector.

It should be worthwhile attempting the stabilization of other enzymes in their active conformation in this or other ways, and characterizing them in terms of kinetics, reaction sequence and stability. Furthermore, the fact that the requirement for soluble effector molecules can be abolished is of value for effector-dependent enzymes of potential practical interest.

Acknowledgements

The authors would like to thank Dr. H. Weetall, Corning Glass Works, for a gift of the glass matrix, Dr. P.-O. Larsson for synthesising the AMP-analogue and Dr. C. R. Lowe for valuable discussion. Part of this work has been supported by the Swedish National Science Foundation.

References

[1] Lindberg, M., Larsson, P.-O. and Mosbach, K. (1973) Eur. J. Biochem. 40, 187–193.
[2] Guilford, H., Larsson, P.-O. and Mosbach, K. (1972) Chemica Scripta 2, 165–170.
[3] Mosbach, K., Guilford, H., Ohlsson, R. and Scott, M. (1972) Biochem. J. 127, 625–631.
[4] Lowe, C. R., Harvey, M. J. and Dean, P. D. G. (1974) Eur. J. Biochem. 41, 347–351.
[5] Brodelius, P. and Mosbach, K. (1973) FEBS Letters 35, 223–226.
[6] Cori, C. F., Cori, G. T. and Green, A. A. (1943) J. Biol. Chem. 151, 39–55.
[7] Fischer, F. H., Heilmeyer, Jr., L. M. G. and Haschke, R. H. (1971) in: Current Topics in Cellular Regulation (Horecker, B. L. and Stadtman, E. R., eds) Vol. 4 pp. 211–251, Academic Press, New York and London.
[8] Dwek, R. A., Griffiths, J. R. and Radda, G. K. (1973) Biochem. Soc. Transactions 1, 617–622.
[9] Axén, R., Porath, J. and Ernback, S. (1967) Nature 214, 1302–1304.
[10] Biomaterial Supports, Corning Biological Products Department, Medfield, Mass. USA, 1973.
[11] Spackman, D. H., Stein, W. H. and Moore, S. (1958) Anal. Chem. 30, 1190–1206.
[12] Bartlett, G. R. (1959) J. Biol. Chem. 234, 466–468.
[13] Larsson, P.-O., Lindberg, M. and Mosbach, K. to be published.
[14] Scott, T. A. and Melvin, E. H. (1953) Anal. Chem. 25, 1645–1661.
[15] Illingworth, B. and Cori, G. T. (1953) in Biochem. Prep. (Snell, E. E., ed) Vol. 3, pp. 1–9, John Wiley and Sons, New York.
[16] Okazaki, T., Nakazawa, A. and Hayaishi, O. (1968) J. Biol. Chem. 243, 5255–5271.
[17] Mott, D. M. and Bieber, A. L. (1970) J. Biol. Chem. 245, 4058–4066.
[18] Shaltiel, S. and Er-El, Z. (1973) Proc. Natl. Acad. Sci., U.S. 70, 778–781.
[19] Gestrelius, S., Mattiasson, B. and Mosbach, K. (1973) Eur. J. Biochem. 36, 89–96.
[20] Feldmann, K., Zeisal, H. and Helmreich, E. (1972) Proc. Natl. Acad. Sci., U.S. 69, 2278–2282.
[21] Hulla, F. W. and Fasold, H. (1972) Biochem. 11, 1056–1061.