AN IMMOBILIZED CYCLIC MULTI-STEP ENZYME SYSTEM – THE UREA CYCLE

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1. Introduction

Several studies of sequentially operating, immobilized enzyme systems have been carried out. The object of such investigations was mainly to find model systems for membrane-bound enzymes or enzymes supposedly operating in close proximity to one another in vivo, as useful information on especially aspects of metabolic regulation may be gained from such studies [1]. Amongst the systems studied, the 2-enzyme system hexokinase–glucose 6-phosphate dehydrogenase [2] and the 3-enzyme system malate dehydrogenase–citrate synthase– lactate dehydrogenase [3] can be mentioned. When immobilized the former system has a far shorter lag phase prior to reaching a steady state (by which the concentration of the rate-limiting intermediate glucose-6-phosphate is high enough) whereas the overall efficacy, i.e., steady state rate, of the latter system is higher (probably because of a shift of the equilibrium of malate dehydrogenase towards oxaloacetate formation caused by the co-immobilized citrate synthase). Similar studies have been carried out on phosphoglucomutase–glucose 6-phosphate dehydrogenase [4] and that of uricase–catalase–allantoinase–allantoicase [5], respectively. In addition, the 2 enzymes, β-glucosidase–glucose oxidase, were immobilized to demonstrate feedback control [6]. In all these cases the immobilized preparations were obtained by simultaneous co-immobilization. It was therefore thought worthwhile to extend these studies and immobilize the enzymes constituting a complete metabolic cycle.

2. Materials and methods

Ornithine carbamoyltransferase (EC 2.1.3.3) (600 U/mg protein from *Steptococcus faecalis*), arginase (EC 3.5.3.1) (55 U/mg protein from bovine liver), inorganic pyrophosphatase (580 U/mg protein from bakers' yeast), myokinase (1000 U/mg protein from porcine muscle), pyruvate kinase (575 U/mg protein from rabbit muscle) and lactic dehydrogenase (550 U/mg protein from beef heart) were obtained from Sigma Chemical Co. Argininosuccinate synthetase (EC 6.3.4.5) was prepared through the bentonite treatment as in [7]. While preparing argininosuccinate synthetase at her institute, a modification of the described method was suggested by Dr S. Ratner. A different $(NH_4)_2SO_4$ fractionation procedure was introduced involving collection of the 0–30% $(NH_4)_2SO_4$-fraction of the liver homogenate for the subsequent purification of argininosuccinate lyase (EC 4.3.2.1), after which the $(NH_4)_2SO_4$-fraction (30–45%) was collected for the preparation of argininosuccinate synthetase. The pH of the liver homogenate had, however, to be adjusted to 5.4 to be able to extract both enzymes simultaneously. The specific activity obtained was 0.65 U/mg protein from steer liver. Argininosuccinate lyase was purified from steer liver according to [8] with the above modification. The specific activity obtained was 10.8 U/mg protein.

One unit is defined here as the amount of enzyme which causes the formation of 1 μmol product/min at 37°C using excess of substrate. Citrulline, arginine, fumaric acid, ornithine, aspartic acid, phospho(enol)-pyruvate, ATP, NADH, urea and carbamoyl phosphate were purchased from Sigma Chemical Co.

Argininosuccinate was enzymatically synthetized from arginine and fumarate and subsequently isolated and purified as the barium salt according to [9]. The content of argininosuccinate was 2.0 μmol/mg Ba salt. 6-Aminohexyl–Sepharose (AH–Sepharose 4B) and Sepharose 4B were obtained from Pharmacia Fine Chemicals.

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2.1. *Analytical methods*

The activity of ornithine carbamoyltransferase was measured at pH 8.5 according to [10]. The activity of the immobilized ornithine carbamoyltransferase was determined by taking aliquots from an Ehrlenmeyer flask containing immobilized enzyme and substrate and assaying them with the above procedure. Argininosuccinate synthetase activity was measured at pH 7.5 with the spectrophotometric method [11]. In this method, the AMP formed is measured with the coupled 3-enzyme system myokinase, pyruvate kinase and lactic dehydrogenase, leading to oxidation of NADH. By spectrophotometrically measuring the formation of fumarate at 240 nm, the activity of argininosuccinate lyase was estimated at pH 7.5 as in [8]. Both immobilized argininosuccinate synthetase and immobilized argininosuccinate lyase were measured spectrophotometrically with the 'stirred batch reactor system', in which the assay mixture after passage over a filter is continuously pumped through a flowthrough cuvette mounted in a spectrophotometer [12].

The arginase activity was estimated at pH 9.5 as in [13]. Immobilized arginase activity was estimated by taking aliquots from an Ehrlenmeyer flask containing immobilized arginase and substrate. The aliquots were subsequently assayed with the same assay procedure used for soluble arginase.

2.2. *Coupling to glutaraldehyde-activated aminohexyl–Sepharose*

AH–Sepharose was activated with glutaraldehyde according to [14]. The coupling of enzymes to the glutaraldehyde activated gel was modified as follows: To 3 ml AH–Sepharose (aspirated) were added 6 ml water and 1 ml 25% glutaraldehyde solution (final conc. 2.5%). After being gently stirred for 10 min at room temperature the Sepharose was carefully washed with several volumes of coupling buffer. To 1 ml activated AH–Sepharose (aspirated) was then added 1 ml enzyme solution. The coupling was allowed to proceed 1.5 h at 4°C during gentle shaking. The gel was then carefully washed with cold coupling buffer, 0.5 M NaCl, 0.2 M glycine (pH 8.5) and finally with the assay buffer.

2.3. *Urea cycle experiments*

The urea cycle enzymes were immobilized with the glutaraldehyde activation method. The activation of the AH–Sepharose was made as above. To 2.0 ml activated gel was added 2.0 ml cold enzyme solution. The coupling solution contained ornithine carbamoyltransferase, argininosuccinate synthetase, argininosuccinate lyase, arginase and inorganic pyrophosphatase. Inorganic pyrophosphatase was added to remove formed pyrophosphate, which inhibits argininosuccinate synthetase. The coupling of the enzymes was allowed to take place at 4°C in 0.1 M potassium phosphate buffer (pH 8.0) for 1.5 h. The enzyme gel was subsequently thoroughly washed as above. In order to assay the different enzymic activities the gel was divided into smaller samples. The amounts of the individual enzymes added and the results of the immobilization procedure are given in table 1. After determination of the individual enzymic activities, 1 ml of the 4 urea cycle enzyme-carrying beads was used for measurement of the activity of the total urea cycle. A comparable system of soluble urea cycle enzymes and inorganic pyrophosphatase was set up. This system contained the same total enzymic activities as those expressed by the immobilized system, except for ornithine carbamoyltransferase and inorganic pyrophosphatase. The individual enzymic activities of these 2 enzymes added were equal to the amounts added per ml activated gel in the coupling solution, i.e., 30 units and 25 units, respectively. The total activity of urea cycle enzymes was measured in the same way for the immobilized and soluble systems with the following method: 1 ml urea cycle-carrying beads or 1 ml soluble urea cycle enzyme solution was added to a small Ehrlenmeyer flask containing 10.8 ml 20 mM potassium phosphate buffer (pH 7.5) and 4.15 mM ATP, 8.3 mM $MgCl_2$, 83 mM KCl, 8.3 mM carbamoylphosphate, 8.3 mM aspartate. After equilibration of the solution (11.8 ml) at 25°C for 15 min, 200 μl arginine solution was added to initiate the reaction of the urea cycle. The final arginine concentration in the solution was 250 μM.

2.4. *High performance liquid chromatography (HPLC)*

Aliquots of 100 μl were taken at intervals from the solution and heated to 95°C for 10 min. After cooling and centrifugation of the aliquots they were analyzed by ion-pair HPLC under the following conditions:

Column size	200 × 4.6 mm
Column packing	Nucleosile-C_{18}, 5 μm
Eluent	2 mM *tert*-butylamine in 50 mM phosphate buffer (pH 6.0)–methanol (9:1)

Flow rate	1.3 ml/min
Sample size	20 μl
Detection	UV, 240 nm

The amount of fumarate (retention time 6.4 min) was determined by measuring the peak areas and comparing them with standards. These conditions also made it possible to measure AMP production.

3. Results and discussion

Of the 4 enzymes constituting the urea cycle (fig.1) the 2 enzymes argininosuccinate synthetase and argininosuccinate lyase had to be prepared essentially according to [7,8]. As described in section 2 a modification involving a different ammonium sulfate fractionation step was introduced, while N. S. was purifying the enzymes at Dr S. Ratners' institute. Subsequently, a number of alternative immobilization procedures for the urea cycle enzymes were tested, including entrapment in polyacrylamide and covalent attachment to Sepharose, either with the cyanogen bromide activation procedure in analogy to work on the same enzymes in [15,16] or with coupling to glutaraldehyde treated 6-aminohexyl-substituted Sepharose. As the latter procedure in our hands proved to give the most active preparation of the most labile enzyme, argininosuccinate synthetase, 25% recovery of enzymic activity, compared with 0.6% for CNBr-activated Sepharose or 3% for polyacrylamide entrapment, this procedure was used for all subsequent immobilization. The 4 enzymes of the urea cycle together with inorganic pyrophosphatase were co-immobilized to the same beads. The individual enzyme activities had to be determined before preparation of the 'cocktail' of the corresponding soluble system. This was done as in section 2 and included addition of either substrate in excess. The arginase activity was assayed following urea production (spectrophotometric), argininosuccinate synthetase was assayed through formation of AMP with the coupled enzyme system, and argininosuccinate lyase was assayed following fumarate production (spectrophotometric). As seen from table 1 the recovery of the different enzyme activities after coupling to the glutaraldehyde treated 6-aminohexyl-substituted Sepharose for 1.5 h, varied from 12–67%. Immobilized ornithine carbamoyltransferase was not determined for the co-immobilized preparation, but in a separate experiment it was shown to retain as much as ~70% of added activity (21 units). For the soluble system 30 units were used – this implies vast excess of this enzyme in relation to the others. The soluble and co-immobilized urea cycle enzyme systems were then assayed after initiation with arginine. Aliquots were taken at intervals (fig.2) and the amount of fumarate formed was determined with ion-pair HPLC. The system developed was very convenient as it allows rapid determination of small quantities of fumarate (linear range 10 μM–5 mM) as well as simultaneous determination of the AMP formed. It is clear from fig.2 that the immobilized

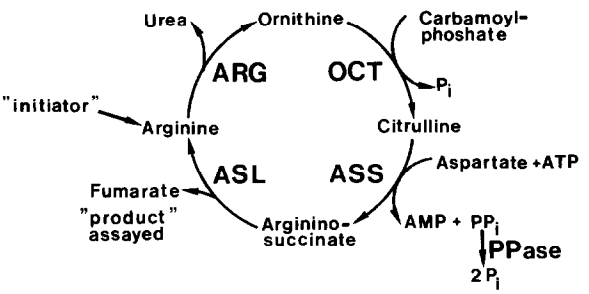

Fig.1. Urea cycle: OCT, ornithine carbamoyltransferase; ASS, argininosuccinate synthetase; ASL, argininosuccinate lyase; ARG, arginase; PPase, inorganic pyrophosphatase.

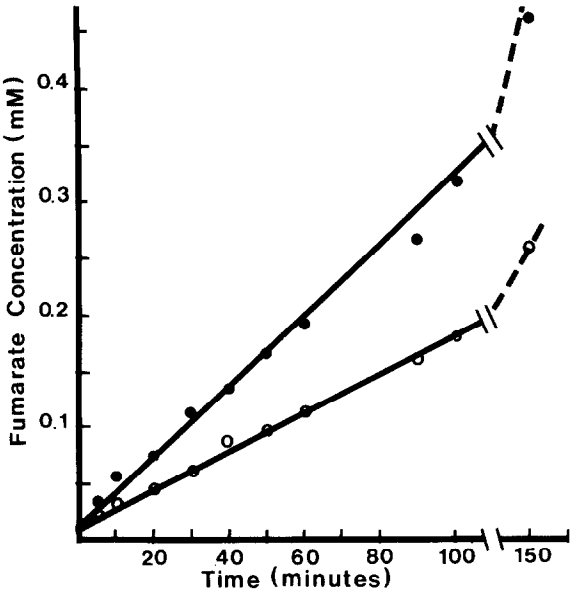

Fig.2. Formation of fumarate with an immobilized (•) and soluble (○) urea cycle enzyme system, respectively. The cycle was initiated with 250 μM arginine.

Table 1
The separate enzymic activities of the co-immobilized urea cycle enzymes

Enzyme	Added enzyme activity at 37°C (units/g aspirated gel)	Immobilized enzyme activity at 37°C (units/g aspirated gel)	Yield (%)	pH in assay solution	Immobilized enzyme activity at 25°C and pH 7.5 (units/g aspirated gel)	Cycle ratio of immob. *versus* soluble system at pH 7.5 and 25°C
Argininosuccinate synthetase (0.65 units/mg protein) K_m-citrulline = 2×10^{-4} M	4.0	0.71	18	7.5	0.31	
Argininosuccinate lyase (10.8 units/mg protein) K_m-arg. succ. = 2×10^{-4} M	5.4	0.66	12	7.5	0.23	
Arginase (55 units/mg protein) K_m-arginine = 10^{-2} M	12	8	67	9.5	2.2	1.88
Ornithine carbamoyltransferase (600 units/mg protein) K_m-ornithine = 2×10^{-3} M	30	21	70	8.5	9[a]	

[a] Not determined, estimate based on the pH-activation profile for free enzyme

In addition inorganic pyrophosphatase was immobilized to remove pyrophosphate formed (25 units were added/g aspirated gel)

urea cycle system is more efficacious than the corresponding soluble system. Previous studies on immobilized multi-step enzyme systems have shown that these systems are more efficacious in the lag phase, i.e., they reach the steady state level considerably faster than the corresponding soluble system, after which both systems show the same rate of product formation. In the system studied here no difference was observed during the lag phase (assays started after 5 min incubation). However, already after this period the rate of fumarate formation (3.2 μM/min compared with 1.7 μM/min) was substantially higher with the immobilized system than with the soluble system. This higher steady state rate kept constant during the entire assay (150 min). We ascribe this higher efficacy to a higher local concentration of the intermediates in the microenvironment within the beads owing to the close proximity of the participating enzymes and/or diffusional hindrances due to the unstirred layer surrounding the beads. As the amount of fumarate formed was higher than that of arginine added we conclude that the cycle is operative. The number of recyclings of a part of the arginine molecules added may in fact very well be higher than that indicated by the data given in fig.2 since the unstirred layer of the beads might allow only a small fraction of the arginine to diffuse into the immobilized system. Addition of considerably larger amounts of arginine was not possible as this compound is known to act as a competitive inhibitor for the preceding enzyme, argininosuccinate lyase, of which only a relatively small amount was present on the beads. While this work was in progress a report appeared on entrapment of the urea cycle enzymes in fibrin [17] in contrast to the covalently bound system described here. The authors discussed effects similar to those given here. However, as the specific activities of the embedded enzymes have not been measured, we feel that a valid comparison between the immobilized and soluble system is difficult to make.

4. Conclusion

The preparation of an immobilized system consisting of the 4 urea cycle enzymes covalently bound to the same Sepharose particles is described. This preparation was shown to be more efficacious than the corresponding soluble system and to operate in a cycling fashion. Higher cycling rates must await further research. Results of such research including also other metabolic cycles will assuredly shed new light on problems bearing on metabolic regulation. In addition, such systems might prove useful models in enzyme technology.

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References

[1] Mosbach, K. and Mattiasson, B. (1978) Curr. Top. Cell. Reg. 14, 197–241.
[2] Mosbach, K. and Mattiasson, B. (1970) Acta Chem. Scand. 24, 2093–2100.
[3] Srere, P., Mattiasson, B. and Mosbach, K. (1973) Proc. Natl. Acad. Sci. USA 70, 2534–2538.
[4] Shimizo, S. and Lehnhoff, M. (1979) J. Solid-Phase Biochem. 4, 75–123.
[5] Okamoto, H., Tipayang, P. and Inada, Y. (1980) Biochim. Biophys. Acta 611, 35–39.
[6] Lecoq, D., Hervagault, J. F., Broun, G., Joly, G., Kernevez, J. P. and Thomas, D. (1975) J. Biol. Chem. 250, 5496–5500.
[7] Rochovansky, O., Kodowaki, H. and Ratner, S. (1977) J. Biol. Chem. 252, 5287–5294.
[8] Ratner, S. (1970) Methods Enzymol. 17/A, 304–309.
[9] Ratner, S. and Murakami-Murofushi, K. (1980) Anal. Biochem. 106, 134–147.
[10] Nakamura, M. and Jones, M. E. (1970) Methods Enzymol. 17/A, 286–294.
[11] Ratner, S. (1970) Methods Enzymol. 17/A, 298–303.
[12] Mattiasson, B. and Mosbach, K. (1976) Methods Enzymol. 44, 335–353.
[13] Mellerup, B. (1967) Clin. Chem. 13, 10, 900–908.
[14] Cambiaso, C. L., Goffinet, A., Vaerman, J. P. and Heremans, J. F. (1975) Immunochemistry 12, 273–278.
[15] Miura, Y., Miyamoto, K., Raze, J. M. and Urabe, H. (1977) J. Biomed. Mater. Res. 11, 743–754.
[16] Miura, Y., Miyamoto, K., Urabe, H., Nagata, C. and Hane, Y. (1977) J. Biomed. Mater. Res. 11, 755–766.
[17] Inada, Y., Tazawa, Y., Attygalle, A. and Saito, Y. (1980) Biochem. Biophys. Res. Commun. 96, 1586–1591.