0273-2289/97/6701-02—0165$12.25

Adsorptive Immobilization of Submitochondrial Particles on Concanavalin A Sepharose-4B

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Received April 1, 1996; Accepted November 1, 1996

ABSTRACT

Submitochondrial particles (SMPs) prepared from beef liver mitochondria were immobilized on concanavalin A Sepharose-4B (Con A-Sepharose). The process of immobilization was optimized by choosing an appropriate buffer system containing Mn^{2+} and Ca^{2+}. Adsorption of SMPs on Con A-Sepharose was found to be a reversible process, nonelectrostatic in nature, and responsive to the presence of methyl α-d-glucopyranose and α-d-mannose. The involvement of membrane glycoproteins in the adsorption process was thus demonstrated. Further analysis of the data obtained on competition of binding by sugar molecules provided competition constants reflecting the potency of inhibition by each individual carbohydrate. Positive-cooperative interactions for binding to the matrix were observed especially at high concentrations of SMPs. The immobilized preparations were used successfully in continuous catalytic transformations involving succinate-cytochrome c reductase (SCR) an enzyme complex of the inner-mitochondrial membrane. Best results were obtained when such operations were carried out at 37°C.

Index Entries: Adsorption; concanavalin A; continuous operation; submitochondrial particles; succinate-cytochrome c reductase.

INTRODUCTION

In recent years, a large amount of attention has been directed toward the development and commercialization of immobilized biocatalysts

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consisting of enzymes, cellular organelles, and whole cells. Such preparations are generally more stable *(1,2)* and easier to handle than their free forms. Furthermore, they can be repeatedly used in continuous catalytic transformations with specific aims. The choice of a purified enzyme or an enzyme in its natural environment in the presence of other biocatalysts is obviously a decision requiring much delibration *(3)*. In such cases, a specific organelle may be considered as a "partially purified" cellular preparation, containing a relatively higher proportion of a particular enzyme or a multienzyme system, compared with the original whole cell.

One of the obvious advantages in using immobilized subcellular structures is their catalytic potential in multistep reactions *(4–7)*. Another important issue is the fact that purification of enzymes which are integral components of cellular structures, is a complicated task. Normally, even after isolation, specific phospholipids have to be added to the apoenzyme preparation in order to achieve desired catalytic activity. Therefore, immobilization of specific cellular structures containing the required enzymes in their natural microenvironment may provide a very useful strategy.

In this article, we report on the use of submitochondrial particles (SMPs) immobilized on Con A-Sepharose in continuous catalytic operations involving the enzymatic activity of succinate-cytochrome c reductase (SCR), present in the inner mitochondrial membrane. In a previous report, Strasser et al. demonstrated the potential usefulness of this matrix for adsorption of SMPs with retention of the catalytic properties of three mitochondrial enzymes *(13)*.

MATERIALS AND METHODS

Materials

All biochemicals were purchased from Sigma (St. Louis, MO). All chemicals were of analytical reagent grade. The following media-buffer systems were used: medium A, 0.22 *M* mannitol, 0.07 *M* sucrose, 10 m*M* EDTA, 0.5 mg/mL bovine serum albumin (BSA), 2 m*M* HEPES (pH = 7.0).

Preparation of Bovine-Liver Mitochondria and SMPs

Mitochondria were obtained exactly as described previously *(8)*, using medium A and medium B described SMPs were prepared by sonication of mitochondrial suspension using an MSE sonicator at maximum cavitation. For this purpose, mitochondrial suspensions, normally at around 15 mg/mL protein, were sonicated four times for 15 (each) on ice. The rest of the procedure was exactly as described earlier *(8)*. Protein was estimated by the Markwell method *(9)*.

Assay of SCR

SCR was assayed according to the procedure of Mackler et al. *(10)*. The effect of pH on the activity of this enzyme was determined using a

buffer mixture consisting of Mes, Pipes, Tris, and glycine each at 3 m*M* concentration adjusted at the required pH by addition of HCl/NaOH.

Optimization of Adsorption of SMP on Con A-Sepharose

Based on information reported in the literature on the requirement for certain ions for optimal interaction between Con A and sugar residues *(11,12)*, different buffer systems were compared. These were: Tris-acetate buffer, 25 m*M*, pH 7.4 (buffer A) used by Strasser et al. *(13)* in a study similar to the present report, Tris-acetate buffer containing 1 *M* NaCl, 1 m*M* $CaCl_2$, 1 m*M* $MnCl_2$ and 1 m*M* $MgCl_2$, pH 7.0 (buffer B), acetate buffer (0.1 *M*, pH 6.0) containing 1 *M* NaCl, 1 m*M* $CaCl_2$, 1 m*M* $MnCl_2$ and 1 m*M* $MgCl_2$ (buffer C) and HEPES buffer (5 m*M*, pH 6.5 to 7.0) containing 1 m*M* $CaCl_2$ and 1 m*M* $MnCl_2$ (buffer D).

Effect of Sugars on Adsorption of SMPs to Con A-Sepharose

200µL batches of Con A-Sepharose suspension corresponding to 100µL of the packet gel and 1.4 mg of lectin were washed twice with 1 mL of buffer D. To the pellet, 100µL of the buffer containing various concentrations of α-D-mannose or methyl α-D-glucopyranose were added. At this point, 100µL of SMP suspension, 5mg/mL protein in buffer D (devoid of sucrose), was added. The suspensions were then mixed by rotation at 4°C overnight. After centrifugation and washing of pellets with buffer D, protein and enzyme activity measurements were carried out.

Continuous Catalytic Operations with Immobilized SMPs

One mL of Con A-Sepharose suspension corresponding to 0.5 mL of the packed gel and 7 mg of lectin was washed with the HEPES buffer described earlier. To the packed gel obtained by centrifugation, 0.5 mL of the SMP preparation containing 15 mg/mL protein was added and the final suspension was mixed by rotation for 2 h at 4°C. The mixture was then transferred to a jacketed column with an internal diameter of 1 cm. The same procedure was followed with fluidizedbed reactors and the set-ups depicted in Figs. 1A, B were utilized.

RESULTS

Experiments were initially carried out to characterize conditions in which SMPs would possess sufficient SCR activity. Thus, the effect of salt and pH on the activity of free SCR was first determined (Figs. 2 and 3). This was followed by determination of the catalytic activity of SCR in different buffer systems and the effect of such media on adsorption of SMPs on Con A-Sepharose (Fig. 4). These buffer systems have been used by various workers in their studies on the interaction of concanavalin A to glycoproteins.

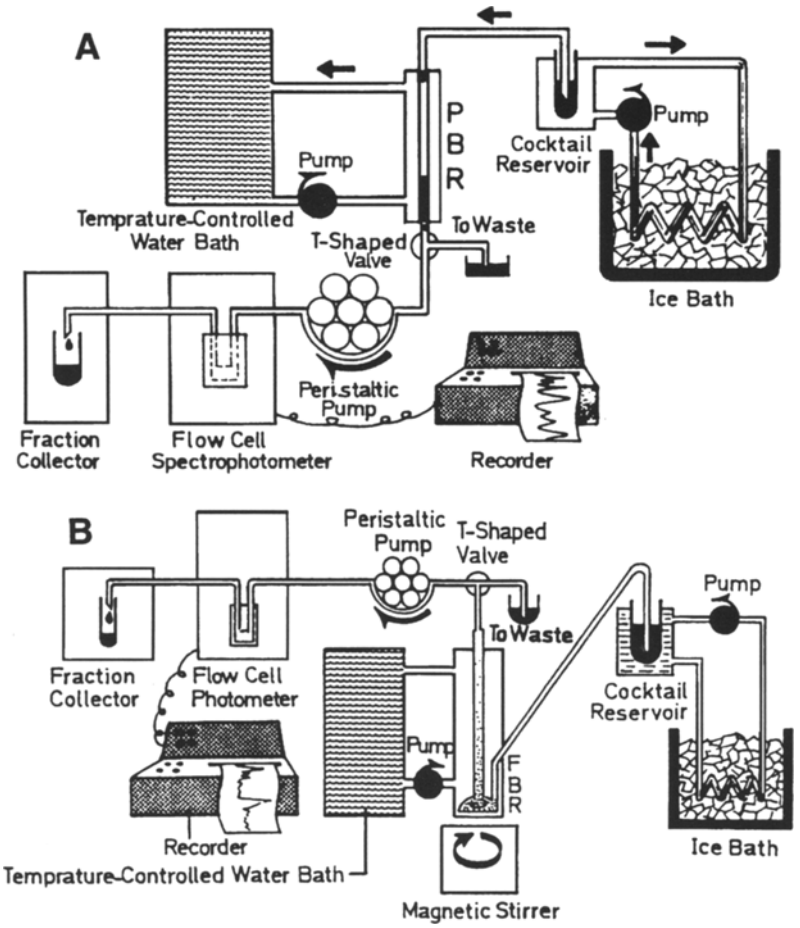

Fig. 1. Flow scheme for continuous catalytic transformation involving the SCR activity of SMPs immobilized on Con A-Sepharose, **(A)** using a packed-bed reactor (PBR) and **(B)** using fluidized-bed reactor (FBR). Further details are described in the Materials and Methods section.

These previous observations guided us in choosing buffer D at pH 7.0. It should be emphasized that an outstanding difference between this medium and a report on a similar use of Con A-Sepharose *(13)* is the fact that it contains Ca^{2+} and Mn^{2+} which are needed to provide the required conformation of the lectin molecule for sugar binding *(12)*. Under such optimized conditions, SMPs aggregated on the beads of Con A-Sepharose-4B could be observed by light microscopy (Fig. 5).

The extent of binding of SMPs to a constant amount of Con A-Sepharose in the form of suspension was investigated. Figure 6 shows the relationship between the amount of SMPs adsorbed (as indicated by enzyme activity) and total concentration of these particles expressed as SMP protein. Owing to interference of the buffer system with protein assay, it was found

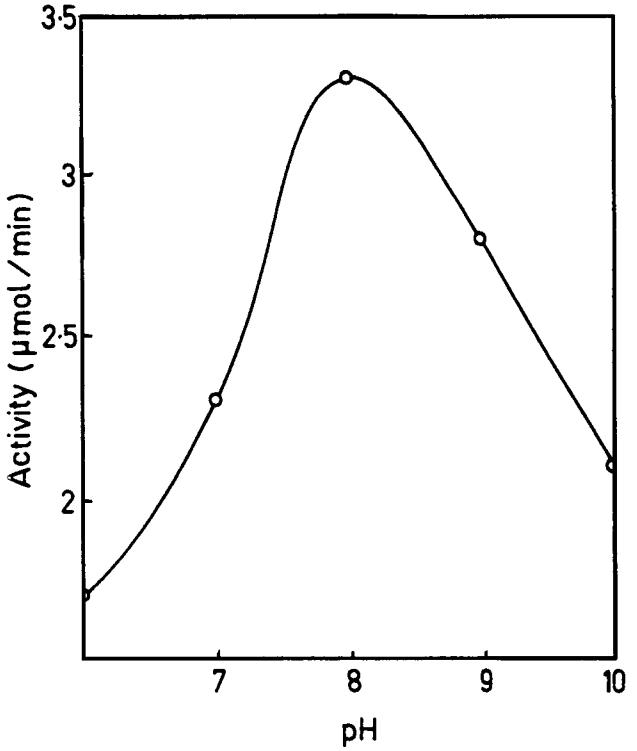

Fig. 2. Effect of pH on the activity of succinate-cytochrome c reductase of free SMPs. The assay medium contained all the required components prepared in mixture of Mes, Pipes, Tris, and glycine each at 3 m*M* concentration adjusted to different pH values.

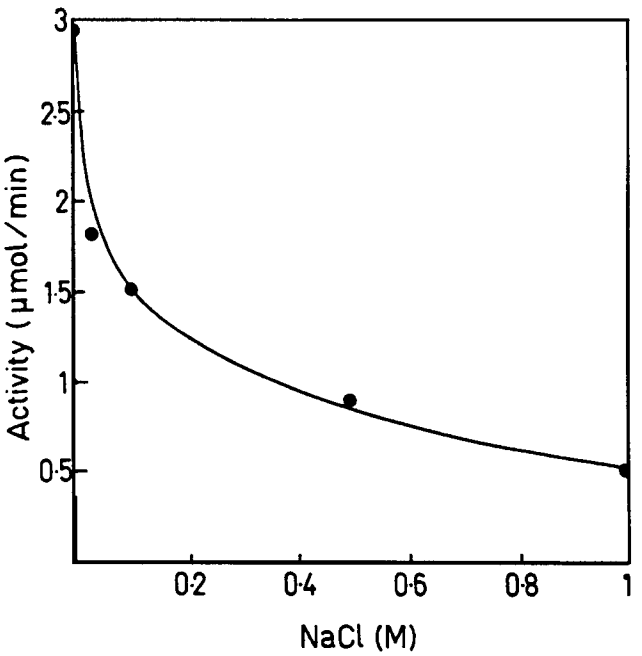

Fig. 3. Effect of NaCl concentration on the activity of succinate-cytochrome c reductase of free SMPs. The assay medium containing different concentrations of NaCl at a final pH of 7.0.

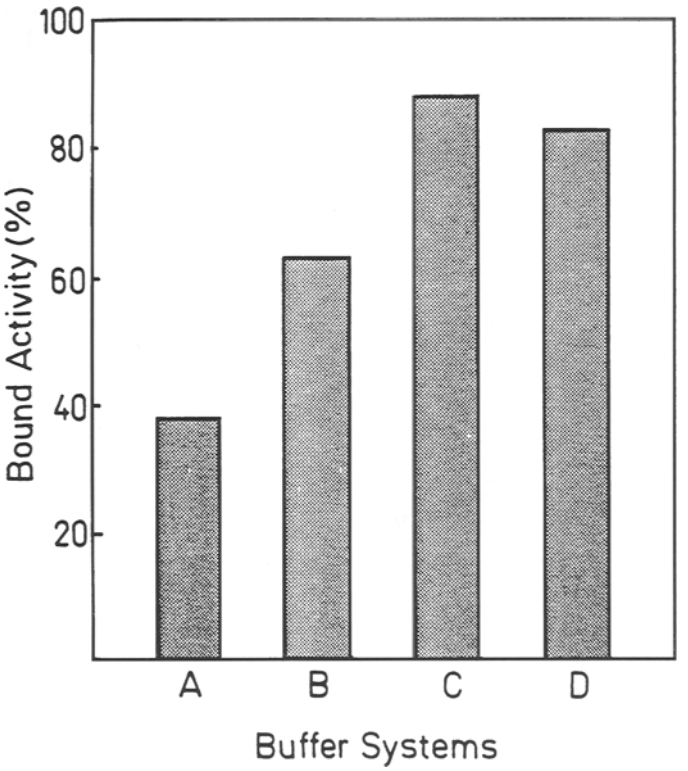

Fig. 4. Adsorption of SMPs on Con A-Sepharose in different buffer systems. 0.5 mL batches of Con A-Sepharose suspension corresponding to 0.25 mL of the packed gel and 3.5 mg of lectin were washed twice with 1 mL of the chosen buffer (A, B, C, and D), as described in the Materials and Methods section, and finally suspended in 0.5 mL (final volume) of the corresponding buffer. To each of these, 200 µL of SMPs containing 2 mg/mL protein was added. The mixtures were mixed by rotation for 8 h at 4°C. The pellets obtained by centrifugation were again washed three times and activities determined. Further details are as described in the Materials and Methods section.

more convenient to determine enzyme activity instead of protein concentration when Con A-Sepharose was used. As indicated, saturation of binding sites on the Con A-Sepharose particles occurs at high concentration of SMPs. Houghs-Klotz, and Hill plot analyses of the data indicate pronounced positive cooperative interactions between each individual SMP for binding to Con A-Sepharose, especially occuring at high concentrations of the particles (Fig. 7). All results reported in this chapter were repeated at least three times and representative data are provided unless otherwise indicated.

Effect of Salt on Adsorption of SMPs on Con A-Sepharose

NaCl was used at various concentrations and its effect on the biospecific adsorption of SMPs on the matrix is depicted in Fig. 8. As shown, no

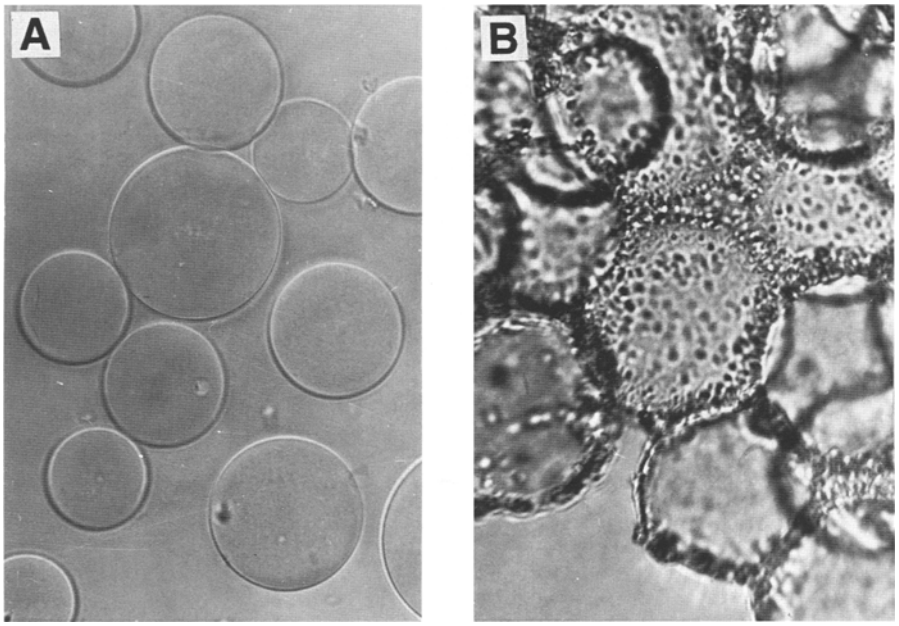

Fig. 5. Light micrograph of **(A)** Con A-Sepharose (×100) and **(B)** immobilized preparation of SMP (×100). Further details are described in the Materials and Methods section and the legend to Fig. 4.

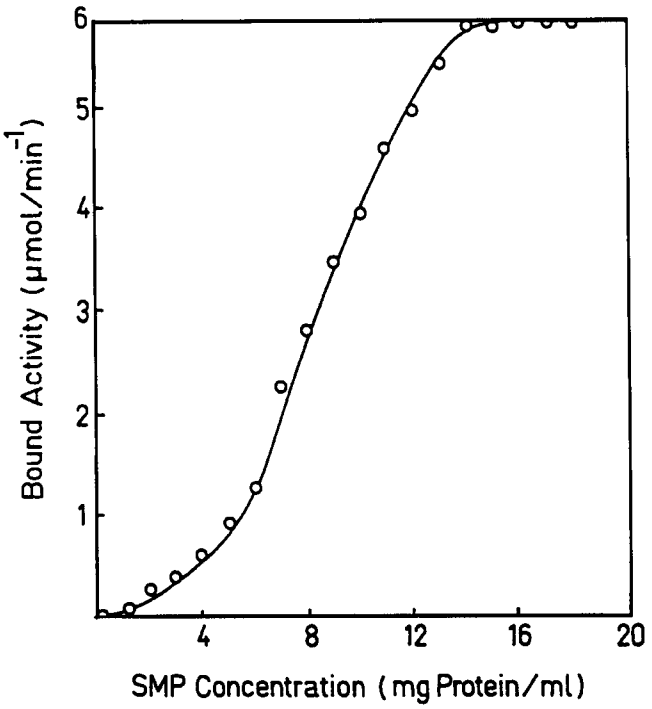

Fig. 6. Adsorption of SMPs on Con A-Sepharose as a function of SMP concentration. 28 mg of Con A-Sepharose totally dried under vaccum was incubated with different concentrations of SMPs in buffer D. After mixing by rotation for 2 h at 4°C, the pellets were washed twice using buffer D and bound activities were estimated.

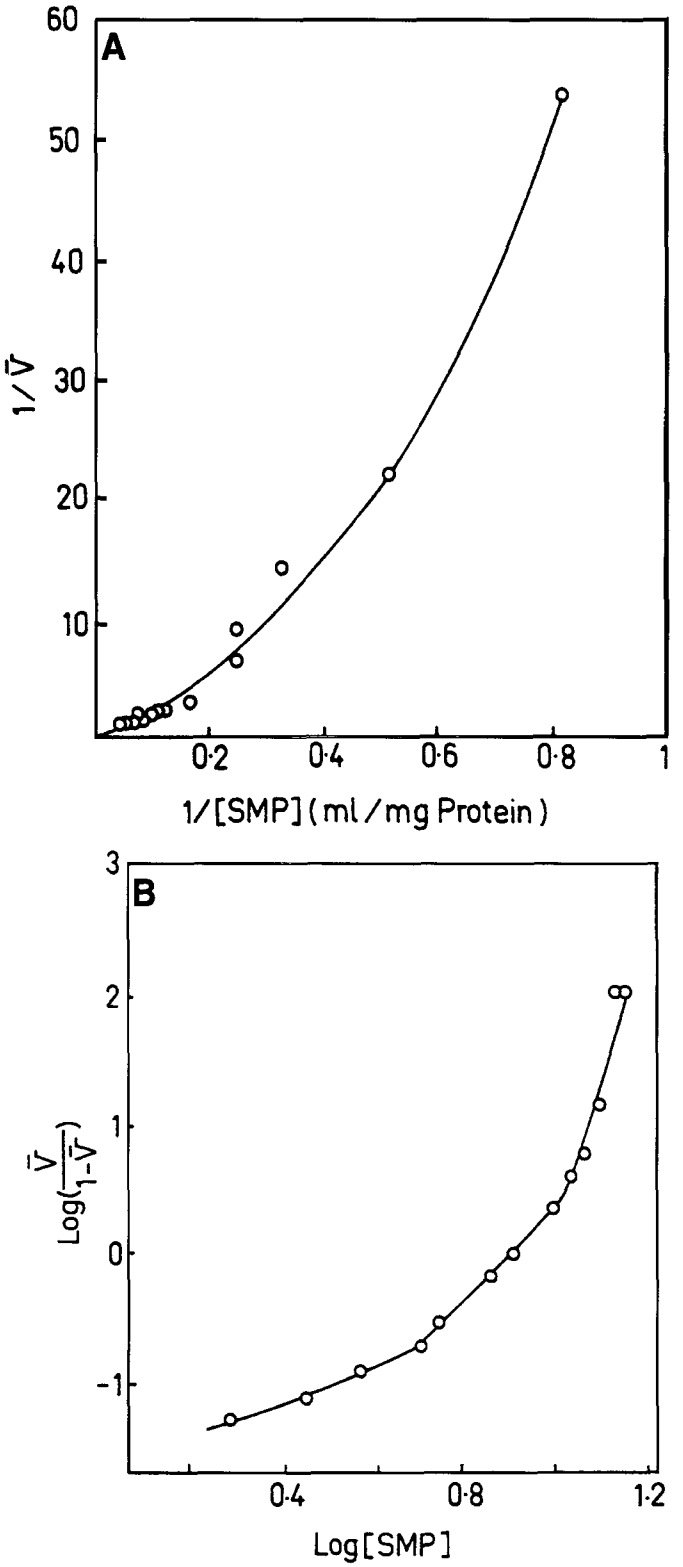

Fig. 7. Houghs-Klotz **(A)** and Hill plot **(B)** analyses the data obtained on interaction of SMPs with Con A-Sepharose.

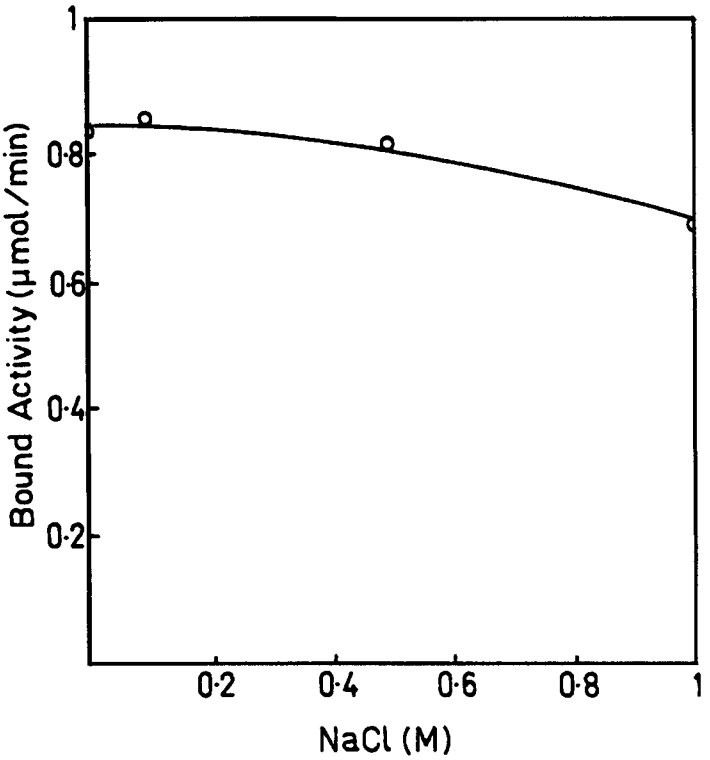

Fig. 8. Effect of NaCl on adsorption of SMPs on Con A-Sepharose.

dramatic effect was observed supporting the notion that interaction between the sugar moieties of glycoproteins and Con A-Sepharose is mainly of a nonelectrostatic nature *(11,14)*.

Effect of Sugars on Adsorption of SMPs on Con A-Sepharose

The specificity of Con A for saccharides was first established by Goldstein and colleagues *(15,16)*, who utilized a variety of simple sugars and oligosaccharides as inhibitors of Con A binding to dextrin. They observed that for interaction to occur, the saccharides must have the D-mannose or D-glucose configuration with the α-anomer being preferred to the β and the methylated forms being more effective than the nonderivatized structures *(17)*. Subsequently, this biological molecule was classified as a mannose/glucose-binding lectin *(18)*.

In this study, we chose α-D-mannose and methyl α-D-glucopyranose to investigate their effectiveness on inhibiting the extent of adsorption of SMPs on Con A-Sepharose. Results presented in Fig. 9 clearly indicated methyl α-D-glucopyranose being more effective than α-D-mannose thus confirming the already published data previously discussed on the general specificity of Con A for saccharides. In line with this observation, methylated α-D-glucose

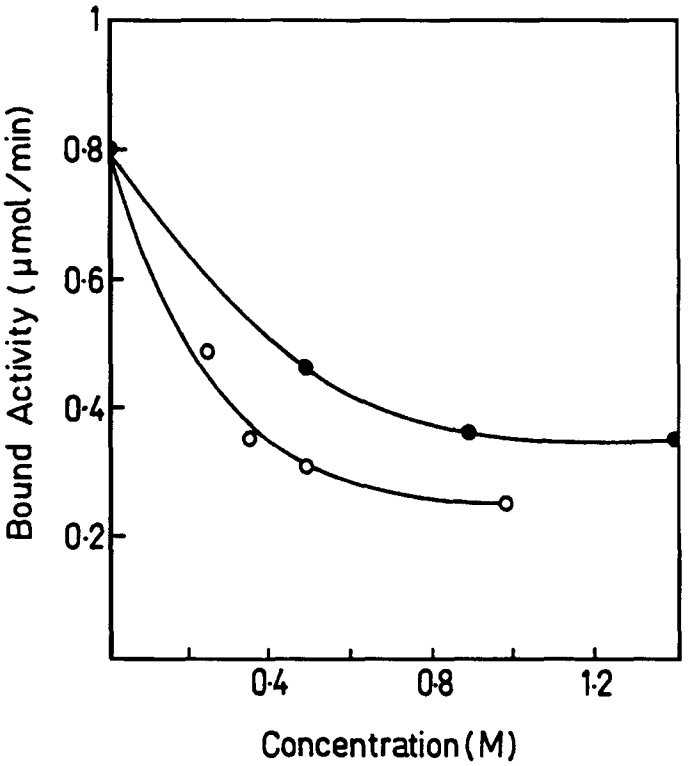

Fig. 9. Effect of α-D-mannose (●) and methyl α-D-glucopyranose (○) on inhibition of SMP adsorption on Con A-Sepharose. Details exactly as described in Materials and Methods section and text.

was more effective than α-D-mannose on releasing SMPs previously bound on Con A-Sepharose (Fig. 10). α-D-galactose, an irrelevant carbohydrate in this regard, was not at all effective (Fig. 10).

Data on this reversible-binding process were further analysed in an attempt to define an inhibition constant. For this purpose, association constants for binding of SMPs or sugar molecules onto Con A-Sepharose particles, all molecules of which are presumed to possess identical binding capacity for a carbohydrate entity, are defined as Ka_1 and Ka_2 as follows:

$$Ka_1 = \frac{[\text{Con A} - \text{SMP}]}{[\text{Con A}] \cdot [\text{SMP}]} \tag{1}$$

and

$$Ka_2 = \frac{[\text{Con A} - \text{Sug}]}{[\text{Con A}] \cdot [\text{Sug}]} \tag{2}$$

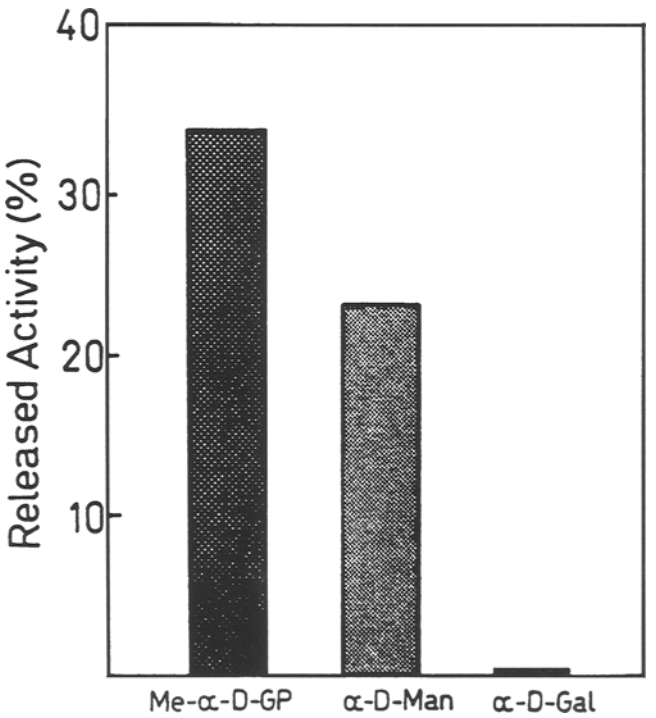

Fig. 10. Effect of various sugars upon release of SMPs adsorbed on Con A-Sepharose. After adsorption of SMPs on the lectin matrix, (the details of which are described in the Materials and Methods section), to pellets containing bound SMPs, 100 µL of a solution of each of the above—mentioned sugars at 1 *M* concentration prepared in buffer D was added. After 10 min incubation at 4°C, followed by centrifugation, activity determinations were carried out in the usual manner.

In which [Con A] is the concentration of unoccupied Con A-Sepharose, [Sug] and [SMP] are the concentrations of free sugars and SMPs. [Con A-SMP] or [Con A-Sug] are concentrations of complexes formed between Con A-Sepharose and SMPs or sugar molecules, respectively. At a constant SMP concentration, we may have a competition constant, K_c defined as:

$$K_c = \frac{Ka_1}{Ka_2} = \frac{[\text{Con A} - \text{SMP}][\text{Sug}]}{[\text{Con A} - \text{Sug}][\text{SMP}]} \tag{3}$$

By defining t as the total number of available sites on Con A-Sepharose for binding carbohydrates and n as the number of sites actually occupied by SMPs and free sugar we may write:

$$[\text{Con A}] + [\text{Con A} \cdot \text{SMP}] + [\text{Con A} \cdot \text{Sug}] = t \tag{4}$$

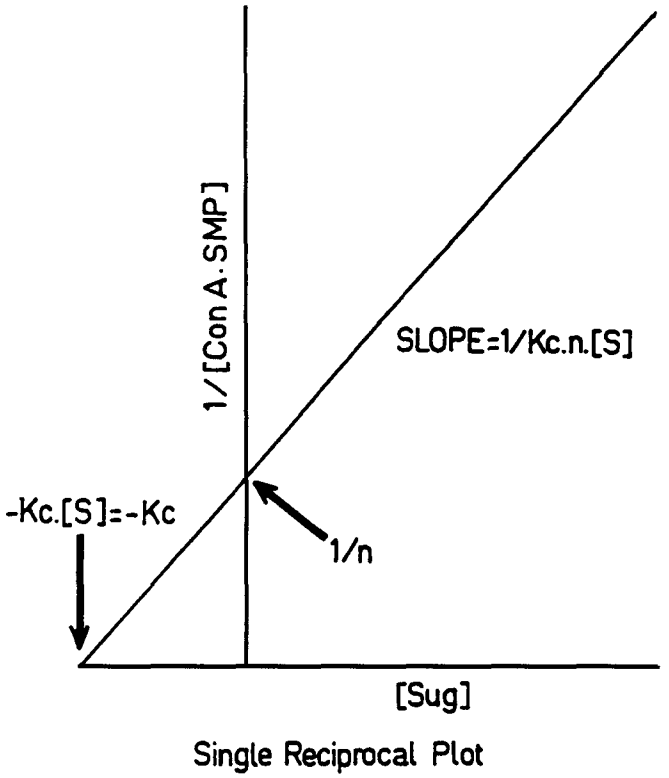

Fig. 11. Single reciprocal plot for the determination of K_c, K'_c, and n. Further details are described in the text.

$$[\text{Con A} \cdot \text{SMP}] + [\text{Con A} \cdot \text{Sug}] = t - [\text{Con A}] = n \quad (5)$$

$$[\text{Con A} \cdot \text{Sug}] = n - [\text{Con A} \cdot \text{SMP}] \quad (6)$$

By substituting n – [Con A · SMP] for [Con A · Sug] in Eq. 3 we may have:

$$K_c = \frac{[\text{Con A} - \text{SMP}][\text{Sug}]}{(n - [\text{Con A} - \text{SMP}] \cdot [\text{SMP}]}$$

or

$$\frac{1}{[\text{Con A} \cdot \text{SMP}]} = \frac{1}{n} + \frac{1}{Kc \cdot n} \times \frac{[\text{Sug}]}{[\text{SMP}]}$$

A plot of 1/[Con A · SMP] against [Sug] is depicted in Fig. 11 where the intercept on the horizontal axis is the apparent competition constant

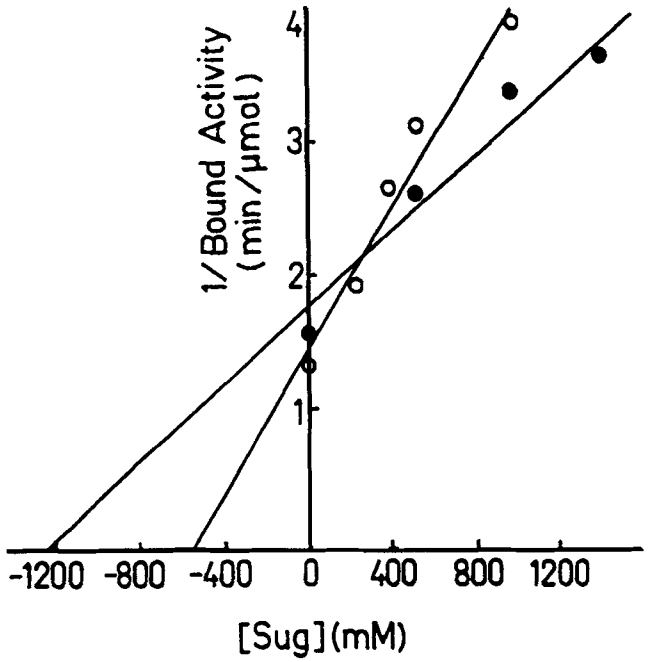

Fig. 12. Single reciprocal plot on competition between SMPs and sugars for adsorption on Con A-Sepharose, α-D-Mannose (●) and methyl α-D-glucopyranose (○) were used at various concentrations as indicated. Details exactly as described in the Materials and Methods section and the text.

(K'c) referred to at a constant concentration of SMP and the intercept on the vertical axis is $1/n$.

Such analysis of the data obtained on competition of binding of SMPs on Con A-Sepharose by α-D-mannose and methyl α-D-glucopyranose are presented in Fig. 12.

As expected, a lower competition constant is obtained in the case of methyl α-D-glucopyranose as compared with α-D-mannose by this analysis.

Immobilized SMPs in Continuous Operations

Figure 13 shows use of immobilized SMPs in continuous catalytic operations at different temperatures. As indicated, the catalytic transformation involving SCR may be achieved using a packed-bed or a fluidized-bed reactor (PBR or FBR, respectively). In both cases, best results were obtained when the catalytic operation was carried out at 37°C (Fig. 14).

DISCUSSION

This article describes the use of a matrix containing Con A for adsorptive immobilization of SMPs with the aim of exploring the catalytic potential of a very active biological membrane. SCR, an enzyme complex of the

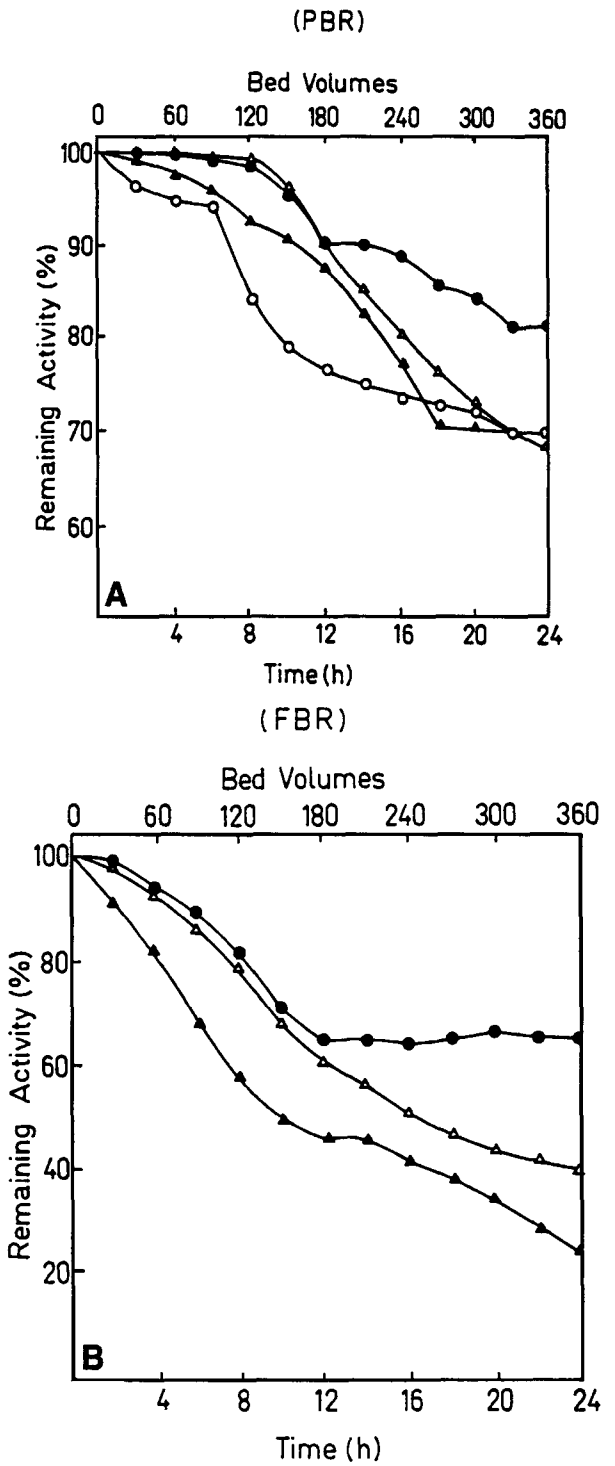

Fig. 13. **(A)** Continuous catalytic operation at 4°C (○), 25°C (△), 37°C (●), and 45°C (△), using immobilized SMPs in the form of column (packed-bed reactor). **(B)** Continuous catalytic operations at 25°C (△), 37°C (●), and 45°C (△), using immobilized SMPs in the form of suspension (fluidized-bed reactor). For further details see Materials and Methods section.

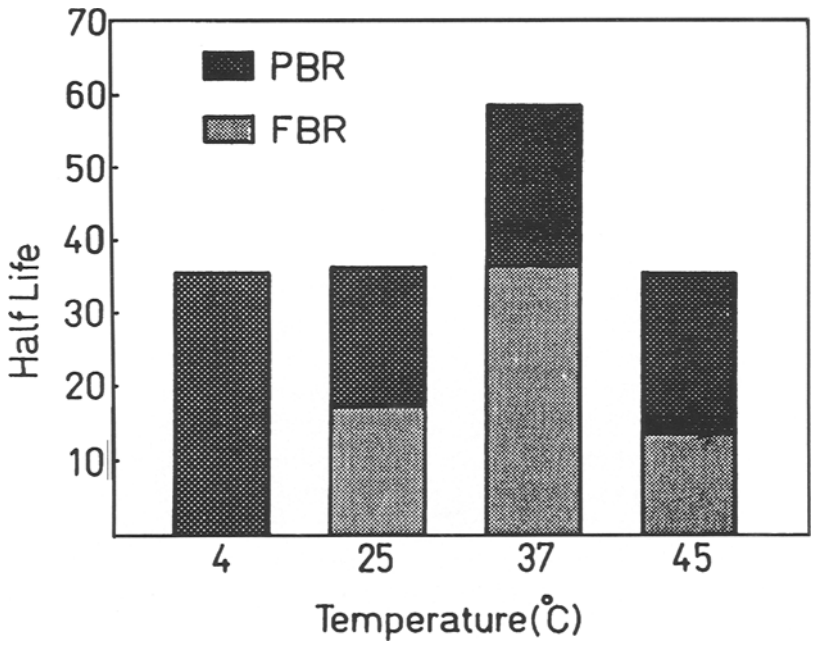

Fig. 14. Histogram indicating the dependency of half-life of activity on temperature in continuous operations using the results presented in Fig. 13 corresponding to packed-bed (▮) and fluidized-bed (▮) reactors.

inner mitochondrial membrane, was chosen as a representative system, especially in relation to multistep reactions carried out within a membrane organization. An obvious advantage in using such membrane structures is the fact that enzyme purification an otherwise very demanding task, is not necessary, considering that apoenzymes extracted from biological membranes may aggregate under normal conditions *(20–22)* and frequently require specific phospholipids *(13,23)* at defined concentrations *(24)* for activity. On the other hand, the direct use of a relatively pure membrane-enzyme preparation, easily obtainable by fractionation of a cellular homogenate, would avoid these inherent complications.

Lectins are a class of proteins that can combine with sugars rapidly, selectively, and reversibly *(25)*. They are defined as carbohydrate-binding proteins other than enzymes or antibodies *(26)*. These proteins have been found to be extremely useful reagents for the detection and isolation of glycoproteins *(14)* showing high specificities for sugar binding equal to or better than that shown by antibodies for their corresponding antigens *(8)*. This unique property of lectins in one hand and the presence of glycoproteins in mitochondria on the other *(28)* has prompted us to use Sepharose 4B substituted with Con A for adsorptive immobilization of SMPs prepared from beef-liver mitochondria.

In the present study, Ca^{2+} and Mn^{2+} were included in the medium, and have been reported to be needed for the maintenance of sugar-binding capacity of Con A *(14)*. Under such optimized conditions, we have observed positive cooperative interactions occuring in the process of binding of SMPs to the matrix. Such preparations were found useful in continuous catalytic operations involving this multienzyme system present in the inner mitochondrial membrane.

Inhibition of binding of SMP to the matrix caused by α-D-mannose and methyl α-D-glucopyranose (Fig. 9) demonstrate that the process of adsorption is indeed via interaction of sugar moieties present in the mitochondrial membranes and Con A particles, as would be expected for a lectin molecule. Reversibility of binding was further confirmed by the observation that the SMPs already attached to the matrix may be released upon addition of carbohydrates to the immobilized suspension (Fig. 10). Analysis of the data on competition of binding by sugar molecules indicated that it is possible to define competition constants from experimental results, which will reflect the potency of inhibition.

The effect of temperature on the stability of SCR activity warrants more discussion. As indicated in Fig. 14, the immobilized preparations remained active for a longer period of time upon increasing temperature from 4°C to 25°C, and best results were obtained at 37°C. It should be pointed out that enzyme stability in free SMPs decreases dramatically by increasing the temperature from 4°C to 45°C. Such results are similar to previous findings on the effect of temperature on stability of purified enzymes immobilized on hydrophobic matrices *(29)*. In both cases, enzyme activity is lost much faster at low temperatures (such as 4°C), presumably owing to insufficient interactions. The importance of hydrophobic interactions in the association of glycoproteins with Con A has already been recognized *(30)*. It should be pointed out that SCR was chosen as a marker enzyme for SMPs whose activity determination was easily possible. It therefore provided a tool for following binding of SMPs to Con A-Sepharose.

In conclusion, results presented in this article report on adsorptive immobilization of SMPs prepared from beef-liver mitochondria on Con A-Sepharose. The process involves specific and reversible interaction between sugar moieties of the mitochondrial membranes and the lectin molecules. By optimization of such interactions, it is possible to obtain preparations useful in continuous catalytic transformations.

Given a large number of commericially available lectins and the presence of carbohydrates on various cells and organelles, the method may provide a useful procedure for adsorptive immobilization of such biocatalysts.

ACKNOWLEDGMENT

Financial support for this work was provided by the Research Council, University of Tehran, Iran.

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