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AFFINITY CHROMATOGRAPHY OF *MYO*-INOSITOL OXYGENASE FROM RAT KIDNEY BY MEANS OF AN INSOLUBLE D-*GALACTO*-HEXODIALDOSE DERIVATIVE

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SUMMARY

Following partial acid hydrolysis, Sepharose 6B-CL was treated with galactose oxidase, leading to an insoluble matrix containing 3-O-substituted D-*galacto*-hexodialdose. The latter substance strongly binds to *myo*-inositol oxygenase from rat kidneys, obviously because of its structural relationship to a reaction intermediate. Free apo-monomers, reconstituted iron(II)-containing monomers and fully active reassociated tetramers of the enzyme all interact with the affinity matrix, the degree of affinity increasing in this order. Thermodynamic analysis led to the conclusion that the ligand coordinates directly to the protein-bound iron ions, but this attachment is strengthened by interactions within and between the protein moiety of the oligomeric enzyme. These interactions seem to be essentially hydrophobic.

INTRODUCTION

myo-Inositol oxygenase (E.C. 1.13.99.1) from rat kidney is an interesting enzyme, both because of its reaction mechanism, being a non-haeme-iron-containing internal monooxygenase, and because of its existence in various oligomeric states[1,2]. The substrate, *myo*-inositol, obviously plays a major role in the interaction of monomers and, consequently, in the regulation of the enzymatic activity[3].

We describe here a new method for the purification of this enzyme, based on its affinity towards D-*galacto*-hexodialdose, an isomer of D-*gluco*-hexodialdose, the latter substance showing high potency as competitive inhibitor and being considered a possible reaction intermediate in the course of the oxygenation of *myo*-inositol[4]. Previously, the enzyme has been prepared according to one of two methods, leading either to highly purified, enzymatically active oligomeric holoenzyme, which, however, becomes more or less irreversibly inactivated within a few days, or to inactive apo-monomers, which are stable and can be almost quantitatively reconstituted to enzymatically active enzyme. Even in the latter instance the total yield is relatively

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low, especially with respect to the earlier stages of purification, making desirable the development of faster procedures of purification. Additionally, immobilized D-*galacto*-hexodialdose offers a tool for studying interactions of this possible transition state analogue and the enzyme under favourable experimental conditions.

EXPERIMENTAL

Enzyme preparations

The preparation of *myo*-inositol oxygenase and apo-monomers and the determination of the enzymatic activity of this enzyme were performed as described elsewhere[1,5]. Galactose oxidase (E.C. 1.1.3.9) and horseradish peroxidase (E.C. 1.11.1.7) were purchased from Sigma (St. Louis, MO, U.S.A.) and Böhringer (Mannheim, F.R.G.), respectively.

Partial hydrolysis of Sepharose 6B-CL

Portions of about 10 g of wet Sepharose were gently shaken with 25 ml of hydrochloric acid of various concentrations for various times at different temperatures, filtered off by suction and washed with 0.1 *M* sodium hydroxide solution, 0.3 *M* sodium chloride solution and finally water.

Determination of the concentration of reducing sugars

A variant of the method of Park and Johnson[6,7] was applied. To 0.5 g of wet Sepharose, 0.2 ml of water, 0.3 ml of 0.5 g/l potassium hexacyanoferrate(III) solution and 0.3 ml of a solution of 5.3 g/l sodium carbonate and 0.65 g/l potassium cyanide were added, thoroughly mixed and heated at 60°C for 30 min. After cooling, 1.5 ml of a solution of 1 g/l of sodium dodecylsulphate and 1.5 g/l of ammonium iron(III) sulphate in 0.05 *N* sulphuric acid were added; after 15 min at room temperature the gel was filtered off and the coloured solution was measured at 690 nm against a blank derived from untreated Sepharose.

Oxidation of partially hydrolysed Sepharose 6B-CL

Optimal conditions and oxidation yields were tested with small samples composed as follows: 0.3 g of gel was added to 1.5 ml of 0.1 *M* phosphate buffer (pH 6.1) and 10 μl of peroxidase (Böhringer, type II, ca. 5 mg/ml) and 50 μl of 20 m*M* aqueous guaiacol were added. The reaction was started by the addition of 10 μl of galactose oxidase (*ca.* 100 units/ml) and followed with continuous stirring at 25°C in a Shimadzu double-wavelength spectrophotometer at 436 and 540 nm. Under these conditions, the reaction proceeded for about 30 min at an almost constant rate.

RESULTS

The results of partial acid hydrolysis of Sepharose 6B-CL are summarized in Table I. There is only a narrow range of experimental conditions (acidity, time and temperature) that leads to both a high recovery of beaded Sepharose and a significant increase in the amount of reducing chain ends. Excessive washing of different samples has shown that the increase in reactive aldehyde groups originates from a reduction in the medium chain length rather than from low-molecular-weight products of hy-

TABLE I

PARTIAL ACID HYDROLYSIS OF SEPHAROSE 6B-CL

Concentration of HCl (M)	*Temperature (°C)*	*Incubation time (h)*	*Recovery (%)*	*Reducing sugars (μmol per g of gel)*
10^{-3}	40	2	100	2.21
	40	5	94	2.33
	60	2	94	3.5
	60	6	91	3.6
	60	24	75	3.8
	80	2	82	4.1
	80	5	42	2.43
10^{-2}	40	2	96	2.45
	40	5	98	2.49
	60	2	89	3.9
	60	6	68	3.7
	60	24	25	4.4
	80	2	≈0	–
10^{-1}	40	2	60	2.9
	40	5	35	2.78
	60	2	26	4.3
	60	6	≈0	–
0	–	–	100	1.34

drolysis caught within the gel pores. As can be seen by comparison of Tables I and II, even in untreated Sepharose 6B-CL an appreciable amount of D-galactose residues appears to be unlinked at C-1 and thus capable of being oxidized by galactose oxidase. After partial hydrolysis this amount is further increased, and most of the newly produced reducing chain-ends seem to be fairly readily accessible to larger protein molecules.

The stability of the Sepharose derivatives prepared in this way is reasonably good. Table III shows that storage at 4°C affects neither the physical integrity of the beads nor the amount of the reducing functions to a serious extent.

TABLE II

TIME COURSE AND EXTENT OF OXIDATION BY GALACTOSE OXIDASE OF PARTIALLY ACID HYDROLYSED SEPHAROSE 6B-CL

Time of partial hydrolysis with 10^{-2} M HCl (h)	*Reducing sugars (μmol per g of gel)*	*Oxidized galactose residues at time t (min) (μmol per g of Sepharose)*			
		10	*23*	*47*	*120*
3	3.5	0.45	0.93	1.1	1.5
6	3.9	1.07	2.12	3.2	3.4
18	4.7	1.23	2.28	2.9	3.2

TABLE III

STABILITY OF PARTIALLY HYDROLYSED SEPHAROSE 6B-CL OXIDIZED BY THE ACTION OF D-GALACTOSE OXIDASE

Storing time (days)	*Recovery of beads (%)*	*Reducing sugars (µmol per g of gel)*
0	100	4.2
2	97	4.07
7	98	3.9
28	95	3.5

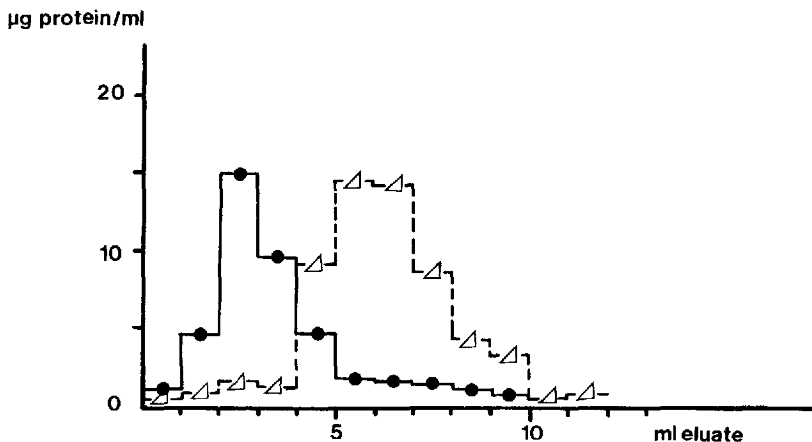

Fig. 1. Chromatography of *myo*-inositol oxygenase monomers on insoluble D-*galacto*-hexodialdose. Columns contained 4 ml of the gel; the flow-rate was less than 6 ml/h. All components were in 50 m*M* Tris-HCl buffer (pH 7.1), 2 m*M* with respect to L-cysteine. (●) 42 µg of apo-monomer in 0.3 ml of buffer applied; (△) as above, with the addition of 20 µl of 10 m*M* iron(II) sulphate solution.

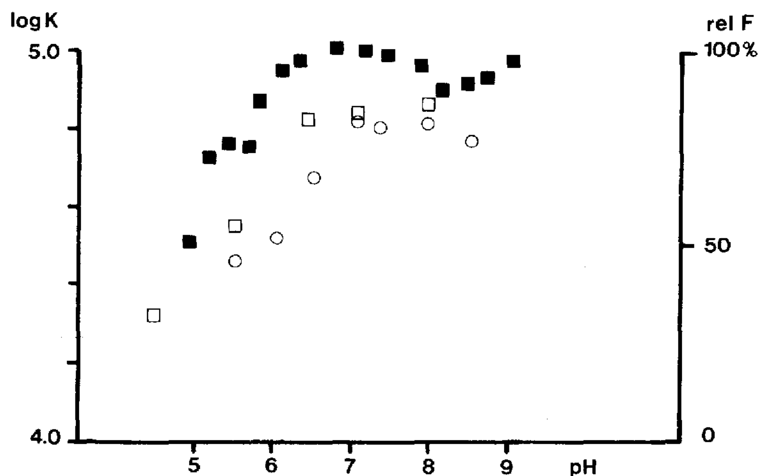

Fig. 2. pH dependence of (○) the affinity constant for binding of apo-monomers to the affinity matrix in the presence of Fe^{2+}; (□) the binding of Fe^{2+} to isolated apo-monomers; (■) the relative intensities (rel F) of the tryptophan contribution to the protein fluorescence of apo-monomers (excitation, 295 nm; emission, 335 nm).

Fig. 1 shows the results of chromatography of *myo*-inositol oxygenase apomonomers on the Sepharose derivative described above. In the absence of iron(II) the enzyme is only slightly retarded relative to "inert" proteins of similar size. On addition of Fe^{2+}, however, monomers bind much tighter to the insoluble matrix. Batchwise experiments led to the evaluation of affinity constants in both instances, namely *ca.* 0.15 M^{-1} in the absence and *ca.* $1.8 \cdot 10^3$ M^{-1} in the presence of Fe^{2+}. The temperature dependence of this behaviour is linear in the range under investigation (277–306°K) and allows the calculation of $\Delta H^0 \approx -3520$ cal and $\Delta S^0 \approx 2.2$ cal/°K. The variation of log K with pH is shown in Fig. 2. It shows a marked resemblance to the corresponding plots obtained for the pH dependence of iron binding and of the intensity of tryptophan fluorescence. It can be concluded from these results that binding and dissociation of this intermediate product analogue is largely governed by the heavy metal ion–apo-protein interaction, and to some extent by apolar interactions within the apoprotein moiety itself.

Fig. 3 shows the chromatographic behaviour of relatively highly concentrated monomer solutions pre-incubated at room temperature with the addition of Fe^{2+} and *myo*-inositol (0.56 and 2 mM, respectively) and thus to a large extent reassociated to tetramers and higher oligomers. With long equilibration times, the oligomers bind strongly to the matrix. Once formed, this complex is strengthened rather than dissociated by the addition of *myo*-inositol, a property in striking contrast to the behaviour of free monomers. Bound oligomers can be competitively eluted by both the product (D-glucuronic acid) and its analogue (D-galacturonic acid) and, although less easily, by free *galacto*-hexodialdose. A quantitative description of this binding and elution behaviour is summarized in Table IV. Whereas the influence of pH and ionic strength on the affinity of oligomeric *myo*-inositol oxygenase towards the affinity matrix under discussion led to the same assumption as with monomers, the temperature dependence of the binding affinity significantly differs from the former situation.

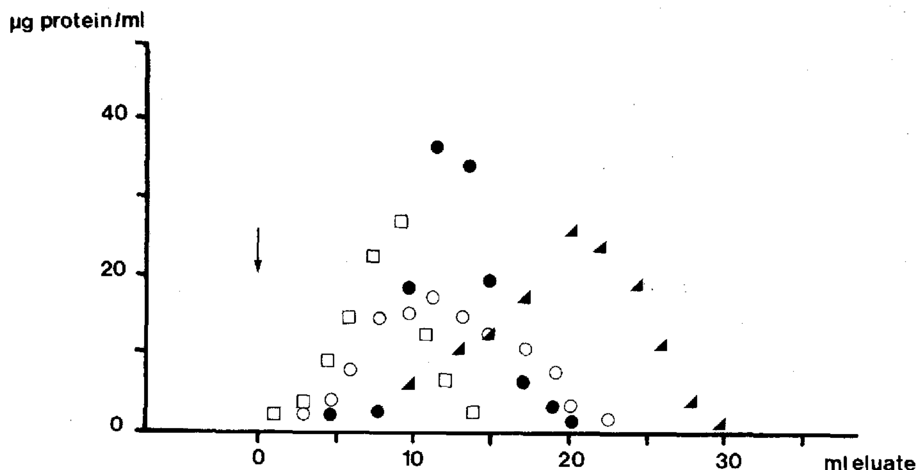

Fig. 3. Column chromatography of reassociated holoenzyme (prepared as described in the text, then applied to a small Sephadex G-25 column; 160 µg of protein per 0.6 ml of buffer) on a 5-ml affinity column. Elution profiles by (□) 2 mM D-glucuronic acid; (●) as above, in the presence of 10 mM *myo*-inositol; (○) 4 M urea; (▲) 4 M urea plus 10 mM *myo*-inositol.

TABLE IV

AFFINITY CONSTANT FOR THE BINDING OF *MYO*-INOSITOL OXYGENASE TO D-*GALACTO*-HEXODIALDOSE-SEPHAROSE

The enzyme was pre-treated as described in the text. The experiments were performed either in columns or batchwise.

Conditions	K_{aff} $(M^{-1} \times 10^{-4})$		
	4°C	*10°C*	*21°C*
No additives	6.2	7.0	8.4
With 1 m*M* *myo*-inositol	6.4	6.9	8.5
W ith 25 m*M* *myo*-inositol	7.1	7.3	8.8
No additions, at pH 5.5	3.0		
No additions, at pH 7.0	6.2		
No additions, at pH 8.5	6.1	6.6	6.8
With 2 m*M* D-glucuronic acid	0.011		
With 2 m*M* D-galacturonic acid	0.04		
With 0.4 m*M* D-*galacto*-hexodialdose	0.12		

The binding of oligomers to the matrix appears to be dependent on the stability of the oligomers themselves, hydrophobic interactions obviously playing a major role in the latter instance[3].

DISCUSSION

As shown by the above experiments, the easily preparable partially oxidized agarose can not only be applied to the recovery and purification of *myo*-inositol oxygenase in high yields, but also provides a means of investigating quantitatively both ligand–monomer and monomer–monomer interactions with this enzyme.

With respect to the first aspect, the affinity matrix offers an absolute minimum of possible undesirable reactivities and affinities and an obviously active site-directed ligand with an affinity significantly larger than various covalently immobilized substrate derivatives[8,9]. The dialdose derivative is less stable than comparable affinity gels, but it can not only be prepared much more easily, but also its oxidation leads

TABLE V

INTERACTION OF *MYO*-INOSITOL OXYGENASE APO-MONOMERS WITH D-*GALACTO*-HEXODIALDOSE WITH ONE OF THE TWO REACTANTS COVALENTLY IMMOBILIZED

Parameter	*Binding of apo-monomers to dialdose matrix*	*Binding of dialdose to CNBr–Sepharose-immobilized apo-monomers*
K_{aff} (M^{-1})	~0.15	~0.2
K_{aff}* (M^{-1})	1.8×10^3	0.6×10^3
ΔH^{0}* (cal)	−3500	−3380
ΔS^{0}* (cal/°K)	2.2	0.55

* 1 m*M* $FeSO_4$ added.

to covalently liganded D-galacturonic acid, a substance that certainly also has some binding power for *myo*-inositol oxygenase. The affinity gel described above, containing D-*galacto*-hexodialdose as an active-site-directed ligand owing to the high concentration of oxidized galactose units and the reasonably strong interaction with at least *myo*-inositol oxygenase holoenzyme, allows quantitative affinity chromatography to be performed. The results support our opinion, originating mainly from binding and kinetic experiments, that active-site-directed binding can be observed even with apo-monomers, but becomes reasonably strong only after prior binding of the catalytically essential Fe^{2+} ion. The affinity is further increased as a consequence of self-association, *i.e.*, by influences leading to increased stability of the oligomers.

Some of these data can be directly compared with analogous data obtained by studying the interaction of unsubstituted D-*galacto*-hexodialdose with covalently immobilized apo-monomers (Table V)[10]. It allows at least an estimate of the extent of deviation from the native behaviour produced by derivatization of either ligands or the polypeptide chain. Even taking into account that the immobilization procedure might lead to subpopulations of matrix-bound enzyme with different behaviour, the protein–ligand interaction appears to be weaker than in the situation reported here. The differences in the derived thermodynamic parameters favour the assumption of at least some loss of conformational flexibility following the protein immobilization.

As under appropriate conditions at least oligomers of *myo*-inositol oxygenase can be practically quantitatively bound to this matrix, this complex will provide an alternative to other methods for studying interactions of the enzyme with various ligands and, perhaps even more informative[11], their kinetics.

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