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SEPARATION OF LIPOAMIDE DEHYDROGENASE ISOENZYMES BY AFFINITY CHROMATOGRAPHY

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Summary

1. Lipoamide dehydrogenase NADH: lipoamide oxidoreductase, (EC 1.6.4.3) from pig heart has been separated into two sets of isoenzymes by chromatography on lipoyl- and NAD^+-derivatized Sepharose-4B matrices. The first fraction is eluted at 30 mM sodium phosphate buffer (pH 7.2), the other requires a higher ionic strength. The two groups originate from the α-ketoglutarate and the pyruvate dehydrogenase complex respectively.

2. Hydrophobic chromatography on a homologous series of alkyl-Sepharoses leads to similar results. The first fraction is eluted with 30 mM phosphate buffer in the case of propyl- and butyl-Sepharose but a high ionic strength is required in the case of an increased chain length (C_5—C_6). The second fraction is reversibly bound on Sepharose-NC_3 and -NC_4 but binding becomes irreversible at higher chain lengths.

3. Aminoalkyl-Sepharoses behave qualitatively as the alkyl derivatives although elution, particularly in the case of the second fraction, can be realized at lower ionic strength.

4. Matrices which are negatively charged (Sepharose-NC_nCOOH, n = 3—7) have no affinity at pH 7.2.

5. The influence of a neutral polar substituent has been studied by comparing the following matrices: Sepharose-NC_6OH, Sepharose-NC_6NH_2 and Sepharose NC_6. Binding of the various isoenzymes is completely reversible in the case of a Sepharose-NC_6OH matrix and the elution behaviour is identical to that on propyl- and butyl matrices.

Introduction

The exploitation of biological specificity as a tool to separate macromole-

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Abbreviation: DTNB, 5,5'-dithiobis-(2-nitrobenzoic acid).

cules has recently received much attention [1,2]. However, the nature of the interactions operative in affinity chromatography has not always been firmly established. Besides the specific interaction between a macromolecule and a suitable matrix-bound ligand other forces of electrostatic and hydrophobic origins may occur. Electrostatic effects can arise from charges which are introduced upon activation of a matrix as occurs for example in the case of cyanogen bromide activation of polysaccharides. Spacer molecules often have a hydrophobic nature. This was observed for instance by Er-el et al. [3], who purified phosphorylase *b* on a Sepharose-glycogen matrix. However, a similar result was obtained on Sepharose which was solely coated with hydrocarbon chains (C_4—C_6). Since then the literature on hydrophobic chromatography has increased considerably [4—9].

Our original intention was to develop an affinity system for the purification of the flavoprotein lipoamide dehydrogenase (NADH: lipoamide oxidoreductase, EC 1.6.4.3). There is no compulsory order in the binding of the two substrates with the enzyme. Therefore, both lipoate- and NAD^+-Sepharose-4B matrices were synthesized. Initial results with the enzyme obtained from pig heart indicated that the enzyme preparation could be separated on both matrices into two main fractions.

An electrophoretic analysis demonstrated that these two fractions correspond with specific isoenzymes of which several are known to occur in mammalian systems (see e.g. ref. 10). However, we will present evidence that this separation is not determined by specific differences in substrate affinity between the various isoenzymes but due to differences in hydrophobic and electrostatic interactions.

Similar results are obtained with alkyl-Sepharoses of different chain lengths (C_3—C_6). Our results indicated that the purification of lipoamide dehydrogenase on propyllipoamide-glass columns as described by Scouten et al. [11] may not be based upon substrate interaction with the active site.

Materials and Methods

Lipoic acid, lipoamide, NAD^+ and other nucleotides were obtained from Sigma Chemical Co., Sepharose-4B from Pharmacia. Dicyclohexyl carbodiimide and the water-soluble 1 ethyl-3/3-dimethylaminopropyl carbodiimide were supplied by British Drug Houses Ltd. and Merck, Darmstadt, respectively; 5,5′-dithiobis-(2-nitrobenzoic acid) was purchased from Aldrich Chemical Co.

Lipoamide dehydrogenase was purified from pig heart as described elsewhere [12—14] and assayed as previously reported [14]. Relatively pure enzyme (absorbance ratio A_{280}/A_{455}, 5.2—6.1) was used for all chromatographical analyses.

Synthesis of alkyl-, ω-aminoalkyl- and ω-carboxyl-agaroses

Sepharose-4B was activated with CNBr (200 mg/g of packed gel) [15]. After the usual filtration and washing procedures the activated Sepharose was suspended in cold 0.1 M sodium carbonate buffer (pH 8.5). Aliquots of 5-ml packed gel were taken to react with the appropriate aminoalkanes, α,ω-diamino alkanes or ω-amino carboxylic acids, each in a final concentration of 20 mM.

The reaction took place overnight at pH 8.5 at a temperature of 4°C under gentle stirring.

After the usual washings with water and high ionic strength buffers to remove unreacted and adsorbed material, the gel was finally taken up in 30 mM sodium phosphate buffer pH 7.2, containing 0.3 mM EDTA. Pasteur pipets were routinely used as columns containing 1—2 ml packed gel.

Preparation of NAD^+- and lipoyl-derivatized Sepharose-4B matrices

Sepharose-4B was activated with CNBr [15] to which ϵ-aminohexanoic acid was linked as a spacer. NAD^+ was coupled in aqueous 80% (v/v) pyridine solution by the dicyclohexyl carbodiimide promoted condensation reaction [16]. The total amount of gel-bound nucleotide was not determined.

The lipoyl derivatives were synthesized as follows: Sepharose-4B was activated as usual with CNBr and either ethylene diamine [15] or L-lysine were coupled. In the latter case, L-lysine was added in a final concentration of 0.05 M at pH 8.5 to an amount of Sepharose-4B equivalent with 30 ml of packed gel. After a 20 h reaction, the derivatized Sepharose gels were eluted with distilled water and taken to pH 4.7 with dilute HCl. Lipoate, 150 mg, dissolved in a small volume of dimethyl formamide was then added, followed by the addition of water-soluble carbodiimide, 200—300 mg/30 ml of packed Sepharose in total, divided over two portions, and added with a 3 h interval. The pH was kept constant throughout the whole reaction (pH 4.7). The amount of lipoate bound was determined by reduction of the lipoyl · Sepharose-4B complex with $NaBH_4$ and determination of the sulfhydryl concentration with DTNB using an extinction coefficient of 13.6 $cm^2 \cdot mmol^{-1}$ at 412 nm [17]. The amount of ligand bound varied between 5.7 and 11 μmol/ml of packed Sepharose.

Glass columns (Pharmacia) with a cooling jacket were filled with Sepharose-4B derivatized beads. The columns were equilibrated with 0.03 M sodium phosphate buffer pH 7.2 containing 0.3 mM EDTA and eluted at a flow rate of 12—18 ml/h, at a temperature of 4°C. Prior to its application on the column, the enzyme was dialyzed against the same buffer used to equilibrate the column. The fractions were analyzed for catalytic activity, protein content and by polyacrylamide gel electrophoresis (on 8% gels) according to Davis [18].

Whenever the protein concentrations were too low, the samples used for the gel electrophoresis were concentrated in a dialysis bag against solid polyethylene glycol. The gels were routinely stained with Coomassie Brilliant Blue.

Results

Chromatography on lipoyl-agarose derivatives

The behaviour of lipoamide dehydrogenase on lipoyl-derivatized matrices was studied with several pig heart enzyme preparations. Since in the α-keto-acid · dehydrogenase complexes the substrate, lipoate, is protein-bound through a lysyl residue [19]. L-Lysine was coupled to the activated Sepharose-4B gel under pH conditions which favour linkage to the matrix through the α-amino group. The lysyl moiety, which has an ϵ-NH_2 group with an approximate pK value of 10.5, was subsequently used to react with lipoate.

Samples of the enzyme, all with an absorbance ratio at 280/455 nm between 5.2 and 6.1, containing 4—5 mg in 0.25—0.35 ml were chromatographed with 30 mM sodium phosphate buffer pH 7.2 containing 0.3 mM EDTA to prevent sulfhydryl groups becoming oxidized which leads in this particular enzyme to a catalytically modified enzyme [20]. The enzyme sample separated visibly on the column into two fractions. This could also be detected by the bright fluorescence visible upon shining ultraviolet light onto the column. The elution profile is shown in Fig. 1A.

The first protein fraction which appears is probably an impurity as it has

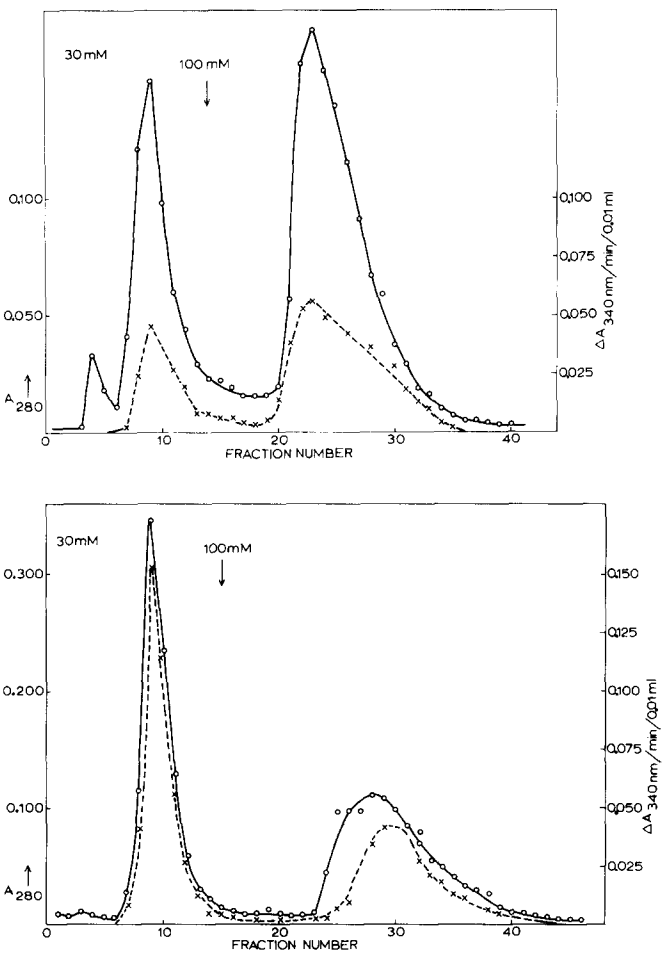

Fig. 1. A. Lipoamide dehydrogenase retention on lysyllipoamideSepharose 4B at 4°C, 4.5 mg of enzyme (A_{280}/A_{455} = 6.1) was applied in 0.25 ml to a 7.0 cm X 1.5 cm column of the matrix. The elution occurred with 30 mM sodium phosphate buffer pH 7.2 with 0.3 mM EDTA and after 26 ml the elution was continued with 100 mM sodium phosphate pH 7.2 with 0.3 mM EDTA. Fractions of 1.8 ml were collected and assayed for absorbance at 280 nm (○———○) and lipoate activity (X———X). B. Lipoamide dehydrogenase retention on NAD^+-Sepharose 4B at 4°C, 5.4 mg of enzyme (A_{280}/A_{455} = 6.0) was applied in 0.35 ml to a 9 X 1.5 cm column of the matrix. Elution as described in Fig. 1A except that fractions of 2.6 ml were collected in this case. Absorbance (○———○) and lipoate activity (X———X) were measured in all fractions. The enzyme used here originates from a different batch than the one used for the experiment described in Fig. 1A.

no catalytic activity and shows no flavin fluorescence. The high absorbance ratio of 6.1 (280/455 nm) in the initial sample also indicates the presence of contaminating material. Bovine serum albumin is eluted in a separate experiment at the same volume as the impurity. The first fraction which contains lipoamide dehydrogenase activity appears later indicating a weak interaction with the matrix, since on a molecular weight basis it would be eluted from the column before serum albumin. The second fraction is retarded considerable. An increase in ionic strength, however, by the addition of potassium chloride (250 mM) or by taking higher phosphate buffer molarities (100—150 mM), also releases this fraction of the enzyme.

In order to verify the specificity of the interaction the influence of substrate on the elution behaviour was investigated. The binary substrate-binding constants are unknown under our conditions. Therefore K_m values were used as an indication for the substrate concentration range needed, although these were determined under somewhat different conditions [11].

However, addition of lipoate (100—1000 μM) or lipoamide (300 μM) to the 30 mM phosphate buffer did not release the second enzyme fraction. Thus it appears that the retention is not caused by a substrate-specific interaction. This is also suggested by the fact that no difference in elution behaviour has been observed regardless of whether lipoate was attached to the matrix through an aminoethyl group or through a lysyl group as a spacer. From previous studies one would expect ϵ-lysyllipoamide to be a better ligand [21].

NAD^+-Sepharose-4B chromatography

Recent publications by Barry and O'Carra [22] indicate that the procedure described by Larson and Mosbach [16] for the immobilization of NAD^+ mainly leads to attachment through the ribosyl hydroxyl groups. Although from these considerations the matrix employed in this study might be less appropriate, we obtained some interesting initial results with our flavoprotein. This matrix proved to be much more effective in separating lipoamide dehydrogenase into two fractions than the lipoyl matrix (Fig. 1B). In the starting buffer at protein concentrations of 2—5 mg, the second fraction even remained on the column at its starting point. We have also used this material for preparative purposes separating 50—60 mg of protein at a time, although the protein moved slowly at such high concentrations.

From the foregoing it seemed, of course, of interest to know whether the coenzyme interaction was also non-specific. From kinetic and spectral data dissociation constants of 40—200 μM are known for NAD^+ binding [23]. Addition of 100—200 μM NAD^+ to the elution buffer proved to be completely ineffective in removing the second enzyme fraction, even after a 3 h preincubation period on the column before the elution. The enzyme could be eluted in a similar way as described for the lipoyl-agarose derivatives.

Chromatography on unsubstituted alkyl-agaroses

The results obtained by affinity chromatography with substrate- and coenzyme-substituted matrices are evidently better explained on the basis of aspecific interactions than on active site-directed specificity. Aspecific interactions can arise from the cyanogen bromide activation and subsequent coupling

TABLE I

Abbreviation	Structure
Sepharose	
-NC_3	—NH—CH_2—CH_2—CH_3
-NC_4	—NH—CH_2—CH_2—CH_2—CH_3
-NC_5	—NH—CH_2—CH_2—CH_2—CH_2—CH_3
-NC_6	—NH—CH_2—CH_2—CH_2—CH_2—CH_2—CH_3
-NC_4NH_2	—NH—CH_2—CH_2—CH_2—CH_2—NH_2
-NC_6NH_2	—NH—CH_2—CH_2—CH_2—CH_2—CH_2—CH_2—NH_2
-NC_6OH	—NH—CH_2—CH_2—CH_2—CH_2—CH_2—CH_2—OH
-NC_3COOH	—NH—CH_2—CH_2—CH_2—COOH
-NC_4COOH	—NH—CH_2—CH_2—CH_2—CH_2—COOH
-NC_5COOH	—NH—CH_2—CH_2—CH_2—CH_2—CH_2—COOH

through amino groups since this may lead to N-substituted isourea products as suggested by the work of Svensson [24]. This then could explain anionic-exchange behaviour of the matrix. In this respect it deserves attention that in the case of DEAE-cellulose chromatography with lipoamide dehydrogenase the literature is consistent in reporting a fraction of enzyme which is eluted at a low ionic strength (e.g. 10—30 mM sodium phosphate buffer) and one eluted at much higher ionic strength values [25—27]. Cyanogen bromide activation of Sepharose-4B followed by a room-temperature treatment of at least several days, results in a matrix which gives no retention at all. However, alternative explanations are possible for our results since besides polar forces between matrix or ligand and protein, hydrophobic interactions may also operate. Since our coupling procedure involves a two-step procedure with the matrix the possibilities for side effects are enlarged. In order to investigate this possibility the behaviour of the enzyme was analyzed on homologous series of both substituted and unsubstituted alkyl agaroses (Table I).

In order to be able to judge the influence of the hydrocarbon chain length, the different alkyl substituents were all reacted with portions of the same batch of activated agarose under identical conditions. The enzyme is separated into two fractions on all alkyl-Sepharoses tested although remarkable differences in the tightness of the binding are found.

Some retention of the first fraction is observed in the case of the propyl derivate. This affinity increases rapidly with chain length and particularly in the case of $n = 5$ and $n = 6$ the enzyme is completely smeared out. An increase in ionic strength is required in those cases to remove the first fraction, but since the enzyme becomes partially denatured we could not verify whether the first fraction was quantitatively removed.

The second fraction smears out over propyl-Sepharose in 30 mM phosphate buffer but forms a sharp band on top of the column in the case of Sepharose-NC_4. This part of the enzyme can still be removed from the column with a linear potassium chloride gradient. A representative elution pattern is given in Fig. 2. In the case of the Sepharose-NC_5 and particularly the Sepharose-NC_6 matrix, however, the binding becomes practically irreversible. An increase of the ionic strength (0.6—1.0 M KCl) broadens the enzyme band on the C_5 matrix.

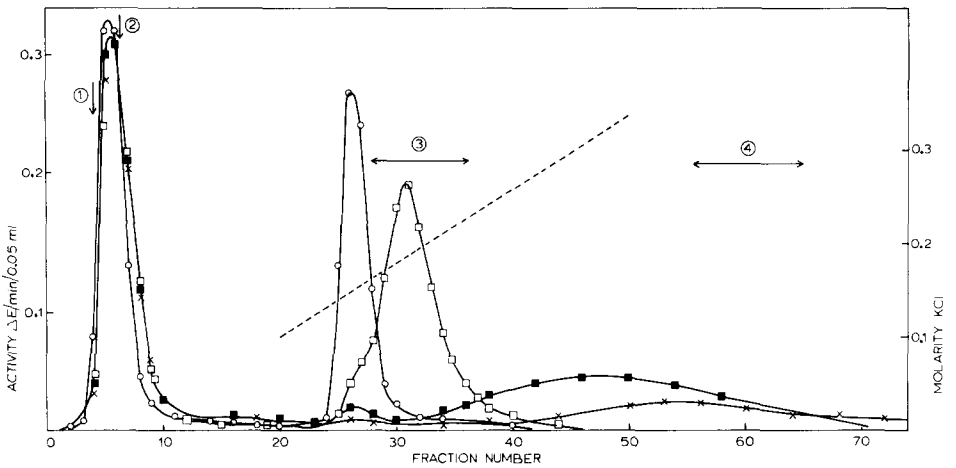

Fig. 2. Elution patterns of lipoamide dehydrogenase on Sepharose-C_4 and Sepharose-C_4-NH_2 matrices. 0.6 mg of enzyme was applied in 0.05 ml phosphate buffer pH 7.2 to a column containing 1.5 ml of packed gel. Elution occurred as in Fig. 1 except that a potassium chloride gradient was applied. Fractions of 10 drops were collected. The symbols correspond with the following experimental conditions. (a) ■———■, butyl-Sepharose 4B at 4°C; (b) □———□, 25°C; (c) X———X, as (a) except for a 15 h preincubation of the enzyme with 0.23% (v/v) *n*-hexane; (d) ○———○, butylamine Sepharose 4B at 4°C.

We have tested several conditions to remove the enzyme from the Sepharose-NC_6; a decrease in ionic strength or addition of oxidized substrates has no effect. The addition of NADH or reduced lipoamide leads to an immediate loss of the yellow colour on top of the matrix indicating that the bound enzyme is catalytically active. Only a very small fraction of the enzyme appears in the eluate, high in diaphorase activity and practically without lipoate activity. After reoxidation in air the yellow fluorescent band reappears on top of the column.

The only possible way to remove the protein quantitatively is by denaturation in concentrated urea or guanidine-HCl solutions.

Since the interaction of the second fraction with butyl-Sepharose is still reversible we have used this matrix to test several compounds of higher chain length with respect to their displacement properties. Hexylamine preincubated in stoichiometrical amounts with the enzyme and added in 50 μM concentration to the elution buffer did not effect the separation. Neither did sodium dodecylsulphate upon preincubation in a 50—100-fold molar excess with respect to the protein. Preincubation of the enzyme with hexane affects the elution pattern of the second fraction unexpectedly since the enzyme becomes even more retarded (Fig. 2).

Chromatography on substituted alkyl Sepharoses

The effect of negatively charged hydrophobic ligands on the separation of the two enzyme fractions has been investigated. Even on Sepharose-NC_7COOH no separation occurs. The actual numbers of residues necessary to eliminate the effect of the carboxylate group has not been determined. Another way to verify whether electrostatic repulsion causes this effect is to lower the pH value of the elution buffer. This indeed leads to retardation of the second fraction.

Since negatively charged groups have such a strong effect on the elution pattern the effect of positive charges was also investigated. Both Sepharose-NC_4NH_2 and Sepharose-NC_6NH_2 were tested. No gross qualitative differences could be observed with respect to unsubstituted alkyl matrices. An extensive analysis was only performed with the Sepharose-NC_4NH_2 matrix. The second fraction could be eluted at a lower ionic strength than on Sepharose-NC_4 (cf. Fig. 2).

The effect of a polar neutral group, viz Sepharose-NC_6OH, on the affinity of both enzyme fractions is remarkable. The binding of both fractions is dramatically changed. The elution pattern is comparable with that on a propyl- or butyl-Sepharose matrix.

Temperature-dependence of chromatography on Sepharose-C_4

It is well known that hydrophobic interactions are strengthened by a rise in temperature. Hjerten [7], however, points out that the effects observed hitherto are small. We have compared the behaviour of lipoamide dehydrogenase on Sepharose-C_4 at two temperatures, viz 4 and 25°C. The results are shown in Fig. 2. The strength of the interactions is weakened contrary to what is expected for hydrophobic interactions.

Electrophoretical analysis of both isoenzyme fractions

The mammalian mitochondria contain several isoenzymes of lipoamide dehydrogenase [25,28—30]. There is evidence that of the 6 main isoenzymes present 3 are derived from the 2-oxoglutarate complex while the other 3 originate from the pyruvate dehydrogenase complex [10,25,28]. Therefore, the fractions separated on the different substrate and hydrophobic matrices were analyzed electrophoretically in order to establish which isoenzymes were involved. As the results shown in Fig. 3 demonstrate the two enzyme fractions indeed correspond with different isoenzymes. The first fraction corresponds with the group of isoenzymes derived from the 2-oxoglutarate dehydrogenase complex while the second fraction originates from the pyruvate dehydrogenase

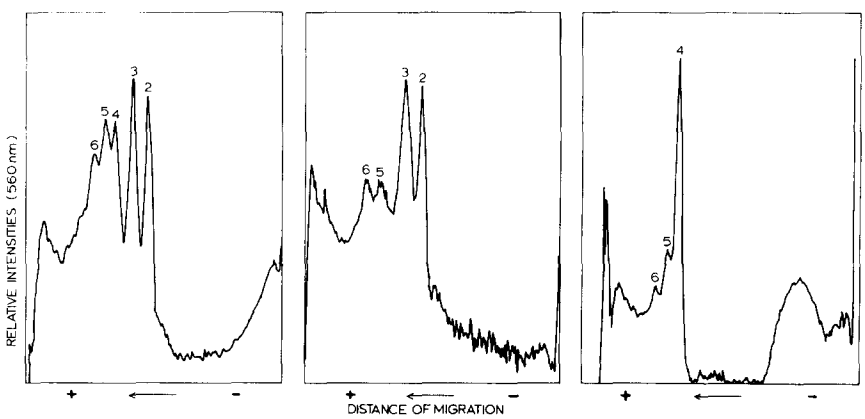

Fig. 3. Scanned disc-gel patterns of lipoamide dehydrogenase separated on NAD^+-Sepharose-4B. From left to right: original enzyme, fraction 10 and fraction 28 (cf. Fig. 1B). Isoenzymes have been numbered from 1 to 6 starting from the cathode. Conditions: gels loaded with 10—20 μg of protein were run in 5 mM Tris · glycine buffer pH 8.2 at 2 mA/tube for 15 min followed by a three-hour period of 4 mA/tube.

complex on the basis of the analyses of Cohn et al. [30] and Kenney et al. [10].

In the case of the NAD^+-matrix it turns out that the first fraction becomes to some extent contaminated with isoenzymes from the pyruvate dehydrogenase complex. For reasons still obscure isoenzymes 5 and 6 seem mainly to be involved. In Fig. 4 some representative patterns are shown obtained with fractions separated on butyl-Sepharose columns (see also Fig. 2). As in some cases the fractions had to be concentrated before electrophoresis, the intensities of the protein bands are only of relative value. The occurrence of denatured protein particularly induced on C_5 and C_6 matrices is clearly illustrated in Fig. 5. The protein band (D) of low electrophoretical mobility which is present in the original preparation only to a minor extent, becomes quite intense on the alkyl-Sepharoses of higher chain length. Furthermore it is clear from the densitograms in Fig. 4 that within one set of isoenzymes differences in retention also occur. The peak ratio of isoenzyme 2 and 3 is not a constant value, for instance over all the fractions within the first enzyme peak. Isoenzyme 4, which is the main component of the second set of isoenzymes, can be obtained separately by taking fractions at the tailing edge of the second enzyme peak (Figs 2 and 4).

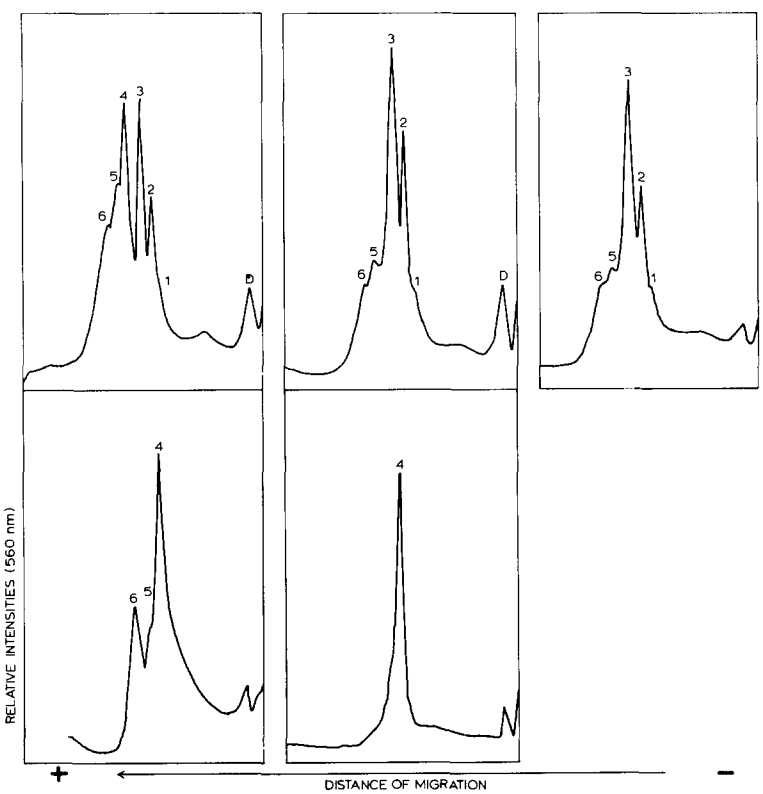

Fig. 4. Disc-gel patterns of lipoamide dehydrogenase fractionated on butyl-Sepharose 4B at 4°C. From left to right: original enzyme and samples from fractions indicated in Fig. 2 (numbers 1—4). For further experimental details see Fig. 3.

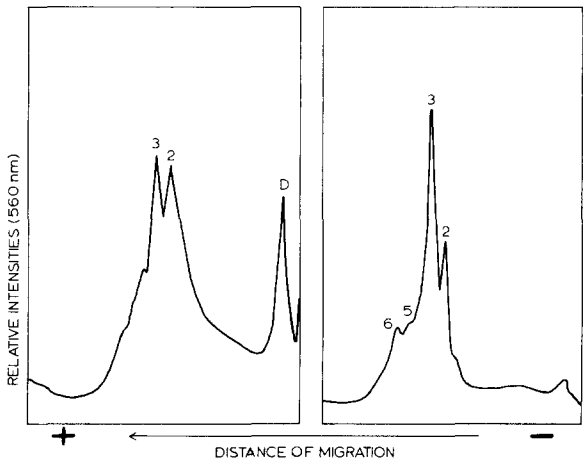

Fig. 5. Disc-gel patterns of lipoamide dehydrogenase fractionated on pentyl-Sepharose 4B at 4°C. The enzyme was initially eluted with 30 mM phosphate buffer (pH 7.2) and subsequently by the stepwise addition of KCl (0.25—0.4—0.6 M). The electrophoretical patterns shown correspond with the first fractions and the enzyme released at 0.4 M KCl. For experimental details see Fig. 3.

Discussion

The affinity of lipoamide dehydrogenase for immobilized substrate and cofactor columns is unlikely to be due to specific substrate interactions alone, since both NAD^+ and lipoamide do not displace the enzyme. The separation into two sets of isoenzymes on these columns is in our opinion mainly due to differences in hydrophobic interactions, since the use of alkyl-Sepharose leads to a similar result. Although it is not clear which parts of the spacer—ligand complexes have to be held responsible for the interaction, our results with alkyl-Sepharose indicate segments of 3—4 carbon atoms to be sufficient to establish a separation. The interaction of the enzyme with propyllipoamide-glass beads as described by Scouten et al. [11] is reminiscent of our own results particularly since in their Fig. 1 the pig heart enzyme seems to become separated into two distinct fractions depending on the ionic strength. However, whether under these conditions displacement of the enzyme can be induced by adding substrate to the elution buffer has not been reported. Neither has an electrophoretic analysis been presented, from which a preferential elution of a set of isoenzymes could be verified.

It is attractive to postulate that the differences in the strength of hydrophobic interactions as observed for the two sets of lipoamide dehydrogenase isoenzymes are functional in their respective multienzyme complexes and represent fundamental differences for the interaction between the flavoprotein and the transacetylase and transsuccinylase respectively. In this respect it deserves attention that in the literature a disintegration of the α-oxoglutarate complex is reported to occur at a urea concentration of 2.5 M [12,31], while one needs 4—6 M urea to dissociate the pyruvate dehydrogenase complex [32]. We have not been able to find kinetic distinctions between the two sets of isoenzymes.

The behaviour of the enzyme on homologous series of substituted and

unsubstituted alkyl-Sepharoses bears many similarities with the work of Shaltiel and Er-el [3,5,8] on glycogen phosphorylase and synthetase. The involvement of specific accessible hydrophobic pockets of the protein is suggested. At higher chain lengths the interactions are stronger and presumably also less specific. Binding therefore, becomes practically irreversible. From our electrophoretical data we conclude that on matrices coated with ligands of higher chain length (C_5—C_6) an increase in protein denaturation occurs.

The system is quite complicated since besides hydrophobic forces, ionic ones may also contribute. This follows simply from the fact that the second enzyme fraction is eluted from Sepharose-NC_3 and Sepharose-NC_4 by an increase in ionic strength, quite opposite to what is expected on the basis of hydrophobic interactions alone. The effect of temperature seems also in conflict with a pure hydrophobic effect (see Fig. 2). In a recent paper by Yost et al. [33] the adsorption is considered to be mainly due to the strong ion-exchange properties of the N-substituted isourea derivatives which are inherent in the coupling procedure used. The hydrophobic ligands strengthen these effects. It might well be that not only positive charge is important but the isourea structure itself. Both are required in this case, as in the case of glycogen phosphorylase [5]. ω-Aminoalkyl derivatives have been found to interact but with a weaker effect than the corresponding alkyl matrices. Explanations have to remain rather descriptive for the moment since the specific sites of interaction are still unknown and so are the salt and temperature effects on the domains of the protein structure which are involved. A change in conditions could lead to changes in accessibility of the binding sites. Modulation of the hydrophobic interactions by introducing other functional groups in the alkyl ligands reveals several interesting points. A negative charge, regardless of the chain length, abolishes the discrimination between the different isoenzymes, whereas positive charges reduce the strength of the interaction (cf. Sepharose-NC_4 and Sepharose-NC_4NH_2), but still lead to separation. Also, in a series of C_6 compounds, viz Sepharose-NC_6, Sepharose-NC_6NH_2, Sepharose-NC_6OH and Sepharose-NC_5COOH, we have observed similar behaviour. Whereas hexyl- and hexylamine Sepharose bind both isoenzyme fractions very strongly, the behaviour of lipoamide dehydrogenase on Sepharose-NC_6OH is similar to that on NAD^+- or that on propyl- and butyl-Sepharose. The introduction of a polar group seems sufficient to make binding reversible. We may speculate on the presence of a negatively charged group at the alkyl-binding site but this requires certainly more firm evidence. Another explanation which we prefer is that the overall negative charge of the protein causes sufficient repulsion to prevent the interaction even with ligands of relatively high chain length.

Whether the individual isoenzymes can ultimately be separated on the basis of hydrophobic chromatography has not yet been investigated in detail. The electrophoretic analysis of different fractions separated on small-sized alkyl-Sepharose columns seems promising (cf. Fig. 4). The ratios at which the isoenzymes within one set are recovered in the different fractions are not identical. Conditions for a complete separation are feasible, particularly in the case of isoenzyme 4.

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