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INVESTIGATION OF ENZYME–SUBSTRATE COMPLEXES BY AFFINITY CHROMATOGRAPHY

APPLICATION TO PIG HEART CITRATE SYNTHASE

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SUMMARY

The relative affinities of Sepharose gels, to which coenzyme A (CoA-SH) and CoA-SH analogues were bound through a well defined site, for citrate synthase were determined. The relative eluting power of coenzyme derivatives for the CoA-SH-gel and the Matrex Gel Blue-bound enzyme was measured, and the influence of oxaloacetate on the binding of the enzyme investigated. From the results, the contributions of different parts of the coenzyme to its binding in the active site and kinetic concepts are derived and found to be in complete agreement with corresponding data for citrate synthase obtained from kinetic measurements reported in the literature. It is demonstrated for some other CoA-SH-specific enzymes that affinity chromatography is of value as an additional tool for the comparative investigation of binding sites of enzymes which depend on the same coenzyme.

INTRODUCTION

Affinity chromatography has become a significant method for the isolation of biomolecules. In addition, it has recently proved useful for investigations on the binding between biomolecules, *e.g.*, enzyme–substrate complexes, and on reaction mechanisms (for a review, see ref. 1). Most previous studies have dealt with ATP- and NAD^+-dependent enzymes, because the substitution and immobilization of the corresponding coenzymes is now well established[2]. However, the binding of coenzyme A (CoA-SH) to polymer supports has only recently been reported, and in some cases evidence for the position of the substitution site is given[3,4]. This is a prerequisite for affinity investigations on binding sites.

By binding CoA-SH and its derivatives through the adenine moiety as well as the SH group, we obtained gels which enabled systematic investigations on the af-

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finity of enzymes to the coenzymes. Preliminary results were reported earlier[5]. The present paper describes the method in detail. Complete results obtained with citrate synthase are given, and discussed in comparison to corresponding data from the literature.

The method involves on the one hand the determination of the affinity of the enzyme to gels with different parts of the coenzyme as affinant and on the other hand the measurement of the competitive displacement of the CoA-SH gel-bound enzyme by coenzyme analogues. From the data obtained the relative contributions of different parts of the coenzyme to the binding in the enzyme–coenzyme complex, as well as the kinetics, can be derived.

MATERIALS AND METHODS

Chemicals

Coenzyme A was a gift from Boehringer (Tutzing, F.R.G.), biochemicals were purchased from Boehringer (Mannheim, F.R.G.) and most of the other chemicals were from E. Merck (Darmstadt, F.R.G.). The Sepharose gels came from Pharmacia (Uppsala, Sweden), Matrex Gel Blue A (ligand Cibacron Blue F3GA) from Amicon (Lexington, MA, U.S.A.), agarose–hexane–coenzyme A-gel Type 5 (S-bound coenzyme A) and 1-N^6-etheno-CoA-SH from P.L. Biochemicals (Milwaukee, WI, U.S.A.).

Analytical methods

Protein determination was performed according to the method of Lowry *et al.*[6], and the evaluation of SH groups by means of Ellman's reagent[7]. The concentrations of free and bound coenzymes or coenzyme derivatives were measured by UV spectrophotometry (Aminco-Chance DW 2 spectrophotometer); the assay of gel-bound substances was performed with the corresponding matrix in the reference cuvette, stirring with a quartz thread prevented sedimentation. The absorption coefficients of bound coenzymes were estimated by determination of the bound phosphate according to the method of Lowry and Lopez[8]. All enzyme activity tests were performed according to established procedures[9].

Syntheses of affinity gels

ε-Aminocaproyl-Sepharose. A 10-ml volume of Sepharose 4B gel (dry weight 0.4 g) was washed with 50 ml of water, then suspended in 10 ml of water. Under vigorous stirring at 10°C, 1 g of BrCN in 0.5 ml acetonitrile was added, the pH was adjusted to 11.0 with 10 *M* sodium hydroxide and then kept constant (pH-stat). During the next 15 min the temperature was raised to 15°C, then the suspension was poured into 100 ml of ice-cold water. The gel was isolated by suction and washed with 200 ml of ice-cold 0.2 *M* sodium bicarbonate solution. It was then added to a solution of 1,3 g (10 mmoles) of ε-aminocaproic acid in 10 ml of 0.1 *M* sodium bicarbonate solution at 4°C, according to the method of Cuatrecasas[10]. After 16 h of shaking, the gel was isolated and washed as indicated in ref. 10.

N^6-Substituted coenzyme gels. A 3-ml volume of ε-aminocaproyl-Sepharose was washed, according to the method of Mosbach and Larsson[11], with 30 ml each of 0.2 *M* sodium bicarbonate solution, 0.01 *M* hydrogen chloride and pyridine–water

(80:20). To the filtered gel in a closed flask, 1.1 mmoles of coenzyme derivative in 6 ml of water and 10.5 g of dicyclohexylcarbodiimide in 25 ml of pyridine were added in aliquots. The mixture was shaken for 10 d at 25°C, and then the gel was isolated by suction and the filtrate was preserved. The gel was rinsed with 50 ml each of water, ethanol, *n*-butanol (40°C), ethanol, water and pyridine–water (80:20). After resuspension of the gel in the reaction medium, 4.5 g of dicyclohexylcarbodiimide were added, and the suspension was shaken for another 10 d at 25°C. The final purification was performed by rinsing the gel as before but omitting the pyridine–water. The yield was determined spectrophotometrically from the corresponding absorption maxima and molar absorption coefficients, *e.g.*, 274 nm and 21,000 M^{-1} cm^{-1} for the N^6-bound coenzymes. In general, between 1.0 and 1.5 μmoles of ligand were bound per ml gel.

Determination of the substitution position of the bound coenzymes. The substitution of adenine derivatives by electrophilic reagents occurs preferably at the 1-position, however, in alkaline solution the derivatives initially obtained are isomerized by a Dimroth rearrangement to the N^6-derivatives[12]. This reaction is accompanied by a typical shift in the absorption maximum, which was also observed during the immobilization of the CoA-SH derivatives. In addition, the amount of free SH groups in the N^6-bound derivatives was found to be equivalent to the amount of the bound coenzyme. Finally, treatment of the gels with a 0.5 *M* solution of dithiothreitol in 0.5 *M* Tris buffer pH 8.5 for 48 h at 25°C yielded adenine derivatives of ε-aminocaproic acid, as shown by mass spectrometric analysis. In the case of the S-bound CoA-SH, no spectral changes and free SH groups were detected. Treatment of the gel as mentioned before yielded S-substituted coenzyme derivatives, and the addition of reducing agents yielded enzymatically active unsubstituted CoA-SH.

Affinity chromatography

Determination of the binding capacities of the gels. A 3-ml volume of the affinity gel in a small column (9 mm I.D.) was equilibrated with 0.01 *M* phosphate or borate buffer pH 7.0 at 4°C (flux 4.8 ml/h); the gel containing CoA-SH was reduced by addition of 10 ml of 0.3% dithiothreitol solution. Then 200 μl of the desalted enzyme solution, containing about twice the amount of enzyme sufficient to saturate the gel, were added. The enzyme activity and the protein content in fractions of 1.6 ml were determined while the gel was rinsed with buffer for 2 h. The capacity of the gel was defined as the difference between the enzyme activities added and those recovered under these conditions.

Determination of the eluting power of coenzyme derivatives. Columns saturated with enzyme and rinsed with buffer as described above were eluted with 10^{-3} *M* solution of the coenzyme derivative for another 2 h (for other concentrations see tables). The enzyme activity eluted was determined and related to the enzyme activity eluted by CoA-SH (= 100) under the same conditions. Non-specific salt elution of the bound enzyme was observed only with salt concentration $\geq 10^{-1}$ *M*. The total recovery of enzyme activity (sum of unbound and eluted activities) was between 85 and 100%. All results are mean values from at least four independent experiments which showed differences of less than 10%.

RESULTS

Citrate synthase

Citrate synthase (E.C. 4.1.3.7) catalyzes the reaction of acetyl-coenzyme A (CoA-S-Ac) and oxaloacetate to give CoA-SH and citrate. The most detailed investigations of citrate synthase have been made with the enzyme from pig heart; it is described as a protein comprising two identical subunits with a total molecular weight of 100,000[13] which needs no cofactor or metal ion[14]. The two subunits have identical active sites and appropriate binding regions for oxaloacetate and CoA-S-Ac[15]. Crystal structure analysis[16] revealed that each subunit consists of a large and a small domain, closely associated with each other.

The stereospecificity of the formation of citric acid[17] indicates that oxaloacetate and the acetyl group of the coenzyme must be firmly bound and orientated in a defined way in the active complex; oxaloacetate is probably bound at the bottom of a pocket close to the acetyl group[15]. The substrate specificity of the enzyme and inhibitor constants of coenzyme analogues[15] lead one to expect that there are special binding areas for the adenine moiety, the 3'-phosphate group and the acetyl group. These results should be confirmed by the affinity experiments.

Binding of the enzyme by different coenzyme affinity gels. Several CoA-SH analogues or derivatives are known to be competitive inhibitors in the citrate synthase reaction[15]. Therefore the affinity of citrate synthase for gels with various ligands was determined and compared to that of the ligands (Table I). The maximum binding capacity (2.3 nmoles enzyme per ml gel) and the ligand density on the gel (1 μmol per ml gel) indicate that one in every 400 molecules of immobilized coenzyme also binds a molecule of enzyme. In comparison with the K_M value of the free ligand (minimum 25 μmoles CoA-SH per ml medium to obtain half saturation), this demonstrates a high and specific affinity between the enzyme and bound coenzyme, especially when one recalls that this binding is observed after a 2-h rinse of the gel with no additional free enzyme in solution.

The gels with the highest capacities are those bearing the ligands with the

TABLE I

AFFINANT BINDING CAPACITIES OF DIFFERENT COENZYME GELS FOR CITRATE SYNTHASE, AND K_M OR K_i VALUES OF THE CORRESPONDING FREE LIGANDS

The capacities are corrected for equal ligand loading of the gels (1 μmol ligand per ml gel). K_M and K_i values from refs. 15, 17–21. Seph = Sepharose; AC = aminocaproyl; Aga = agarose.

Gel		*Ligand*	
Structure	*Binding capacity (nmol enzyme/ml gel)*	*Structure*	*K_M or K_i (μM)*
Seph-AC-N^6-CoA-SH	2.2	CoA-SH	25–130
Seph-AC-N^6-CoA-S-Ac	2.3	CoA-S-Ac	$\leqslant 5$
Seph-AC-N^6-CoA-S-AcAc	2.0	CoA-S-AcAc	80
Seph-AC-N^6-ATP	1.5	ATP	100–700
Seph-AC-N^6-Ade	$\leqslant 0.2$	–	–
Aga-AC-S-CoA	$\leqslant 0.3$	CoA-S-palmitoyl	4

lowest K_M or K_i values; the differences express the relative contributions of the corresponding groups to the binding in the enzyme–substrate complex. The adenine moiety contributes little to the binding except when it bears phosphate groups. A special form of binding may also be postulated for the acetyl group, however, detailed information cannot be derived probably because a nearly total saturation of the gel occurs with all three CoA-derivative gels.

A discrepancy seems to exist in the case of the coenzyme bound through the S atom and the corresponding free substrate with a long chain acyl group. While the latter is a very good inhibitor, indicating strong binding to the enzyme, the gel does not bind the enzyme at all. On the basis of experiments of Caggiano and Powell[22] with spin-labelled long chain fatty acid derivatives of CoA-SH, these compounds are, however, bound at sites different from the CoA-S-Ac-binding site. The difference between the affinity of citrate synthase for CoA-S-palmitoyl (palmitoyl-coenzyme A) and for the insoluble cross-linked carrier bound through a short spacer may thus be due to steric reasons.

The experiments on the eluting power of the coenzyme analogues (Table II) gave more details of the contribution of the adenine moiety of the molecule to the binding in the enzyme–coenzyme complex. The effects of the different compounds were in accord with their inhibitor constants, as far as these were available. The very low efficiency of 3′-dephospho-CoA-SH is indicative of the predominant rôle of the phosphate group in the 3′-position for the binding, while the rather low efficiency of 1-N^6-etheno-CoA-SH may be due to steric hindrance and the additional positive charge at the 1-position of the purine ring.

The eluting power of purine and pyrimidine mononucleotides is high if they bear at least two phosphate residues; even 5-phosphoribosyl-1-diphosphate is a rather good eluent, while AMP, in accordance with its high inhibitor constant (K_i = 6700 μM), has practically no eluting power. Probably, a phosphate group within a certain

TABLE II

RELATIVE ELUTING POWER OF COENZYME ANALOGUES FOR CITRATE SYNTHASE BOUND TO Seph-AC-N^6-CoA-SH GEL

The values given are enzyme activities eluted by a 1 mM solution of the eluent within 2 h (4.8 ml/h), relative to the effect of CoA-SH = 100. K_M and K_i values from refs. 15, 17, 18 and 23.

Coenzyme analogue			*Coenzyme analogue*		
Structure	*Rel. eluting power (CoA-SH = 100)*	*K_M or K_i (μM)*	*Structure*	*Rel. eluting power (CoA-SH = 100)*	*K_M or K_i (μM)*
CoA-SH	100	25–130	ATP	40	100–700
CoA-S-Ac	110	⩽5	dATP	40	200–300
CoA-S-AcAc	90	80	ITP	35	–
3′-Dephospho-CoA-SH	10	–	GTP	30	–
1-N^6-Etheno-CoA-SH	35	–	CTP	20	–
			ADP	18	1400
			AMP	3	6700
			5-Phosphoribosyl-1-diphosphate	21	–

distance of the adenine ring is needed to contact with the site for binding of the 3'-phosphate. These results are in agreement with findings reported in the literature, that NADPH with a 2'-phosphate group is a better inhibitor than NADH[21,23].

Use of a triazine dye in the investigation of the active site. Triazine dyes bound to gels have recently been used for the purification of NAD^+-dependent dehydrogenases by affinity chromatography. Probably because the steric requirements and the charge distribution of these compounds resembles that of NAD^+, they may effect binding to proteins with a "dinucleotide domain"[24]. Even citrate synthase is specifically bound by triazine gels (experiments by P. A. Srere, reported in ref. 24), thus indicating that the size and charge of the active site of the enzyme, at least in part, correspond to the structure of the triazine dyes.

Systematic investigations in our laboratory have shown that Matrex Gel Blue has a high specific binding capacity for citrate synthase form pig heart and that the bound enzyme can specifically be detached by its substrate (Table III). Oxaloacetate proved to be an extremely good and specific eluent, while the competitive inhibitor D-malate[18,25] had no effect. At present this discrepancy cannot be explained. The binding site for oxaloacetate must contain a positively charged group near to where the acetyl group of CoA-S-Ac is located. While CoA-SH and acetoacetyl-coenzyme A (CoA-S-AcAc) itself did not affect the binding of citrate synthase to the triazine gel, they enhanced the eluting power of oxaloacetate. This is in agreement with the finding that the affinity of any of the substrates of citrate synthase is far higher for the binary complex than for the free enzyme. The total ineffectiveness of the combination of oxaloacetate and CoA-S-Ac may be due to their conversion into citrate by the enzyme. The different behaviour of palmitoyl-CoA is in agreement with the existence of an different binding site for this compound and a probable conformational change induced by its binding[22].

The triazine dye Cibacron Blue F3GA and the combination CoA-S-Ac/oxaloacetate are compared in Fig. 1 which shows the striking congruence between their steric requirements and charge distributions, suggesting that the enzyme–dye complex is an analogue of the ternary enzyme–coenzyme–substrate complex discussed previously for other enzymes[24,26,27]. The distance between the two sulphonyl groups on

TABLE III

INFLUENCE OF OXALOACETATE ON THE ELUTING POWER OF COENZYME ANALOGUES FOR CITRATE SYNTHASE BOUND TO MATREX GEL BLUE

The values given are relative enzyme activities eluted within 2 h (4.8 ml/h) by solutions of the coenzyme analogue (1 m*M*) or the coenzyme analogue and oxaloacetate (each 1 m*M*). n.d. = Not determined.

Coenzyme or substrate	*Relative enzyme activities eluted*	
	Without oxaloacetate	*With oxaloacetate*
Oxaloacetate	–	75
CoA-SH	0	100
CoA-S-Ac	0	0
CoA-S-AcAc	0	100
CoA-S-palmitoyl	40	n.d.

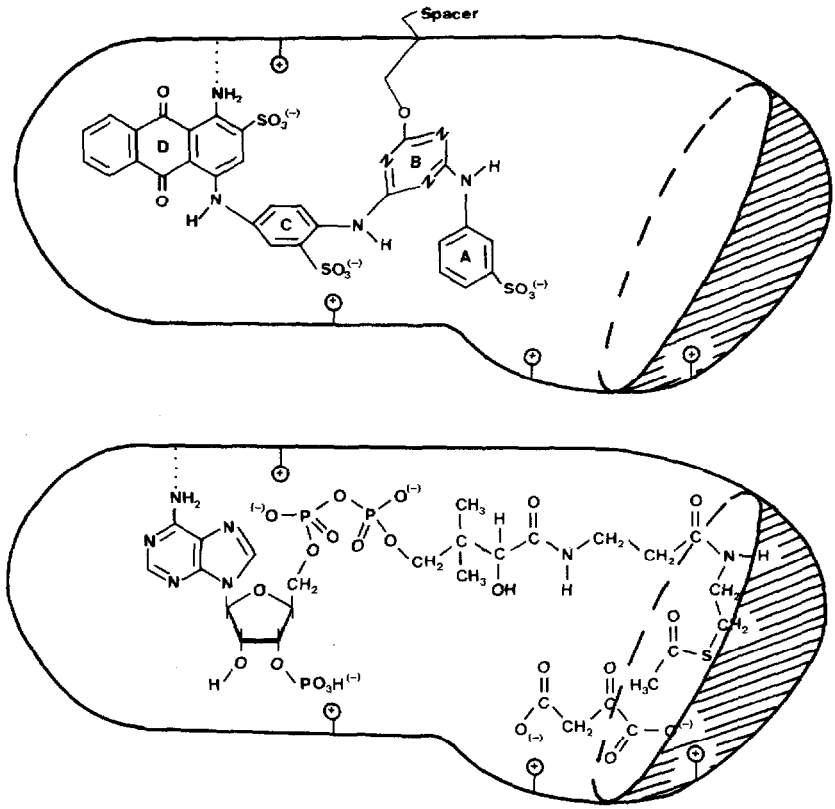

Fig. 1. Comparison of the steric requirements and the charge distributions of Cibacron Blue F3GA (one of the isomers, ligand of Matrex Gel Blue) and of CoA-SH + oxaloacetate. The cleft for the binding of oxaloacetate and the pocket for the acetyl group, as proposed by Srere[15], are indicated.

rings C and A should correspond to that between the sites for the 3′-phosphate of CoA-S-Ac and one of the carboxyl groups of oxaloacetate. From this result, further evidence that the active site is in part a closed pocket may be derived, mainly for the binding region for oxaloacetate and the acetyl group, as already proposed by Srere[15].

Experiments on the binding sequence. In spite of the fact that CoA-S-Ac, even in the absence of oxaloacetate, binds to citrate synthase to form a productive complex, a sequential mechanism with primary binding of oxaloacetate is proposed for the citrate synthase reaction, mainly because the affinity of the enzyme for CoA-S-Ac is enhanced after the binding of oxaloacetate[25].

The binding of the enzyme to CoA-SH gel and its elution by different analogues (Table IV) show that there is a specific affinity of the enzyme for the coenzymes, even in the absence of oxaloacetate. While this substrate by itself did not elute citrate synthase from CoA-SH gel, and did not affect the elution by CoA-SH and CoA-S-AcAc, it significantly enhanced the eluting power of CoA-S-Ac. The weight of this effect is even higher when one takes into consideration that the two substrates are bound to each other by the enzyme, which diminishes their effective concentrations.

TABLE IV

INFLUENCE OF OXALOACETATE ON THE ELUTING POWER OF COENZYME ANALOGUES FOR CITRATE SYNTHASE BOUND TO CoA-SH GEL

The values given are relative enzyme activities eluted within 2 h (4.8 ml/h) by a 0.5 m*M* solution of the coenzyme analogue or by a 0.5 m*M* solution of the coenzyme, containing 1 mmol/l oxaloacetate.

Coenzyme or substrate	*Relative enzyme activities eluted*	
	Without oxaloacetate	*With oxaloacetate*
–	–	5
CoA-SH	40	45
CoA-S-Ac	70	100
CoA-S-AcAc	55	58

The result provides evidence in support of a sequential mechanism[28]. The same conclusion can be drawn from the elution of citrate synthase from the triazine gel (Table III): only oxaloacetate competes with the pseudo-ternary complex for the dye, and the possibility of the formation of other ternary complexes enhances this competition with the dye.

Application of the method to other enzymes

The availability of the affinity gels provided the possibility of using them in preliminary experiments with some other enzymes which also depend on CoA-SH or on coenzyme A derivatives. The experiments mainly concerned the elution by coenzyme analogues of the enzymes bound to CoA-SH gel. Characteristic differences compared to citrate synthase were found.

Thus, phosphotransacetylase (E.C. 2.3.1.8) (reaction: CoA-S-Ac + P $\rightleftharpoons$ CoA-SH + Ac~P) was preferentially eluted by CoA-SH as compared to CoA-S-Ac, which shows that in this enzyme the acetyl group does not contribute to the binding as in citrate synthase. 3'-Dephospho-CoA-SH was 60% as active as CoA-SH, indicating that the 3'-phospho group is not as important for binding as in the case of citrate synthase. This result is in agreement with the high specificity of phosphotransacetylase for CoA-SH[29]. 3-Hydroxylacyl-coenzyme A dehydrogenase (E.C. 1.1.1.35) (reaction: $CoA\text{-}SCOCH_2CHOHCH_3 + NAD^+ \rightleftharpoons CoA\text{-}SCOCH_2COCH_3 + NADH + H^+$) does not possess CoA-SH as a substrate. Accordingly, it was by far the most efficiently eluted by CoA-S-AcAc, demonstrating that there is a special binding region for the side chain, which is to be expected from the sterically unequivocal evolvement of the reduction. Finally, succinyl-coenzyme A synthetase (E.C. 6.2.1.4) (reaction: succinate + CoA-SH + GTP $\xrightarrow{Mg^{2+}}$ CoA-S-succinyl + P + GDP) was best eluted by 3'-dephospho-coenzyme A; from this result, which is in agreement with the fact that this CoA-SH analogue is a substrate for the enzyme[30], one can conclude that the 3'-phosphate group of the coenzyme does not contribute to the binding in the enzyme–coenzyme complex.

DISCUSSION

The affinity chromatographic investigations on citrate synthase confirmed most of the previous results on the binding between enzyme and coenzyme or inhibitors and regulatory molecules reported in the literature: in particular, the relatively unimportant contribution of the adenine ring to the binding, measured previously by kinetic experiments[21], and the evidence for binding of the acetyl group[15,28]. The importance of the phosphate group in the 3′-position[21,23] was demonstrated, the existence of a closed pocket for the thioester group[15] was confirmed and the enhancement of the affinity between enzyme and coenzyme after the addition of the substrate[18,28] and the special binding site for long chain fatty acids[22] were observed. Finally, an application of the method to a comparative study of different enzymes demonstrated its versatility.

It must be pointed out, however, that the results obtained in the present work are still qualitative, as is the case with many of the kinetic experiments. However, this may be in part due to experimental problems which can be overcome. For example, by comparison of Tables II and IV, a change in the concentrations of the eluents results in a change in the relative eluting powers. Thus, a more sophisticated experimental use of this observation could lead to more quantitative results. Another possibility would be to change the elution conditions, *e.g.*, to determine the elution volume for half of the activity bound instead of using a constant elution volume. The most important advantage of the method, however, is that once a gel is prepared, quick comparative investigations can be made with several enzymes, and there is even no special requirements as to the purity of these enzymes, because they would be purified by the affinity chromatography itself.

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