COMPOSITION OF THE CITRATE LYASE ACYL CARRIER PROTEIN FROM *KLEBSIELLA AEROGENES*

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1. Introduction

Citrate lyase from *Klebsiella aerogenes* is a multienzyme complex that contains an acyl carrier protein [1]. In some of its properties this acyl carrier protein is similar to the well known acyl carrier proteins from fatty acid synthetases [2]. For example, the acyl carrier proteins from both enzymes are of similar size (M_r approx. 10 000 [1]) and contain phosphopantothenic acid [1,3,4] residues. They are, however, different proteins which can be clearly distinguished by their different biological activities [1]. It was of interest therefore to determine the composition of the acyl carrier protein from citrate lyase and to compare it with the known composition of those from fatty acid synthetases. Fingerprint analyses indicate that the amino acid sequence of the lyase acyl carrier protein is grossly different from that of the *E. coli* fatty acid synthetase. The most drastic difference, however, was found in the composition and the structure of the prosthetic groups. That of the lyase acyl carrier protein contains not only phosphopantetheine but also adenine, phosphate and sugar residues. All of these residues are covalently linked in the prosthetic group to a substituted isomeric dephospho-CoA. Part of this work has been reported on a fall meeting of the Gesellschaft für Biologische Chemie [5].

2. Materials and methods

[^{14}C]Acetyl acyl carrier protein was obtained in homogeneous form from citrate lyase, purified from *Klebsiella aerogenes* [1]. Molar concentrations of pure acyl carrier protein solutions which were used for phosphate and sugar analyses were calculated from $\epsilon_{260}=18.5\ mM^{-1}\ cm^{-1}$ (see below).

Identical molar concentrations were obtained from protein content [6] using $M_r \simeq 10\,000$ [1] and from β-alanine content determined by amino acid analysis (see below).

3. Results and discussion

3.1. *Amino acid composition of the citrate lyase acyl carrier protein.*

Amino acid analyses were kindly performed by Dr K. Beyreuther on acid hydrolysates of acyl carrier protein samples. The results (table 1) indicate, that the protein is composed of a total of 86 amino acids. These data and the composition of the prosthetic group (see below) indicate a mol. wt of about 10 000. This is the same as estimated from dodecylsulfate gel electrophoresis [1]. The fatty acid synthetase acyl carrier proteins from different bacterial and plant sources are of similar size [2]. Furthermore, the amino acid composition of the lyase acyl carrier protein is similar to those from fatty acid synthetases. The acidic amino acids are in excess, although the aspartic acid content of the lyase is considerably lower than that of the other acyl carrier proteins. The presence of one mol each of cysteamine and β-alanine, which are components of the prosthetic group, is common to all acyl carrier proteins including that from the lyase.

Tryptic digests of carboxymethylated acyl carrier proteins from citrate lyase (*K. aerogenes*) and fatty acid synthetase (*E. coli*) were subjected to fingerprint analysis. No identical peptides were obtained from both proteins. This indicates that the amino acid

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Table 1
Amino acid composition of the acyl carrier protein from citrate lyase.

Amino acid residue	Residues/mol 24 hr hydrolysis	72 hr hydrolysis	Assumed number of residues/mol
Cysteine	0.96	0.94	1
Cysteamine	1.11	1.08	1
β-Alanine	1.29	1.13	1
Aspartic	5.42	5.46	5
Threonine	2.50	2.44	3
Serine	2.63	2.63	3
Glutamic	16.47	17.29	17
Proline	2.01	1.79	2
Glycine	6.51	6.44	6
Alanine	13.03	12.94	13
Valine	8.79	9.11	9
Methionine	2.35	2.38	3
Isoleucine	4.86	4.96	5
Leucine	8.50	8.73	9
Tyrosine	–	–	0
Phenylalanine	0.84	0.88	1
Lysine	3.17	3.31	3
Histidine	–	–	0
Tryptophan	0.65	0.59	1
Arginine	4.00	4.26	4

Carboxymethylated samples were hydrolysed in 4 N methanesulfonic acid containing 0.1% tryptamine [7] at 115°C. Cysteine and cysteamine were determined as the carboxymethyl derivatives.

sequences of both carriers are substantially different. This could explain the observed differences in catalytic activity [1].

3.2. *Adenine content of the acyl carrier protein*

Samples of the acyl carrier protein from citrate lyase had no appreciable absorbance in the visible region but showed a characteristic absorption profile in the ultraviolet (fig.1). Since this spectrum cannot be explained by the absorbance of the two aromatic amino acid residues of the acyl carrier protein (tryptophan and phenylalanine), this result suggested the presence of adenine. This was confirmed by quantitative ultraviolet spectroscopy and by thin-layer chromatography. The spectrum of an acyl carrier protein solution was compared to that of an equimolar solution of adenosine, tryptophan and phenylalanine (1:1:1). The two spectra (320–250 nm) were identical, yielding $\epsilon_{260}=18.5\ mM^{-1}\ cm^{-1}$, and indicating the presence of one mol adenine per mol of the acyl carrier protein. The adenine content is primarily responsible for the observed absorption profile (fig.1). The tryptophan residue contributes about 20% of the total absorbance at 260 nm and is responsible for the characteristic shoulder at 288 nm. The absorbance

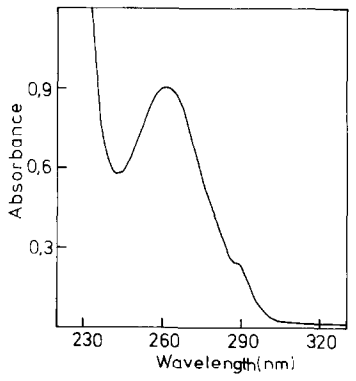

Fig.1. Absorption spectrum of acyl carrier protein in 5 mM potassium phosphate pH 7.0; d = 1 cm; protein concentration 0.5 mg/ml. A Hitachi Model 124 double beam spectrophotometer was used.

Table 2
Adsorption of [^{14}C]acetyl acyl carrier protein to charcoal.

Charcoal added mg	[^{14}C]acetyl acyl carrier protein in the supernatant protein (%)	radioactivity (%)	A_{260} (%)
0	100	100	100
5	53	48	51
10	17	10	20

Charcoal added mg	Serum albumin in the supernatant %	CoA in the supernatant %
10	95	0

A solution of [^{14}C]acetyl acyl carrier protein (43 000 cpm; 29 nmol) in 3 ml of 10 mM K-phosphate pH 7.0 was incubated for 5 min at 25°C with 5 mg of acid washed charcoal. After centrifugation the supernatant was analysed for radioactivity, protein [6] and absorbance at 260 nm. Another 5 mg of charcoal were added to the recombined mixture; incubation and analytical determinations were repeated.
A control solution of serum albumin (3 mg) and CoA (0.02 mg) in 3 ml 10 mM K-phosphate pH 7.0 was treated likewise. The content of the compounds in the supernatant solutions was calculated from the absorbances at 260 and 280 nm.

of the phenylalanine residue is negligible (about 1% of total). Chromatographic identification of adenine was preceeded by hydrolysis of samples containing 50 nmol acyl carrier protein in 0.1 ml of 0.1 N HCl for 1 hr at 100 °C. Adenine was liberated under these conditions and identified by thin-layer chromatography through comparison with an authentic sample.

The presence of one mol adenine in the isolated acyl carrier protein indicated strong, probably covalent linkage between them. Tight binding of adenine to the protein was demonstrated by adsorption experiments. Like other adenine containing compounds the [^{14}C] acetyl acyl carrier protein is readily adsorbed to charcoal (table 2). Adsorption of radioactivity, of protein and of the adenine moiety, all occur to the same extent. In control experiments, mixtures of serum albumin and CoA were separated by the same method; i.e., all of the serum albumin remained in solution, whereas CoA was completely adsorbed.

3.3. *Phosphate and sugar content of the acyl carrier protein*

The prosthetic group of fatty acid synthetase acyl carrier proteins from different sources is 4′-phosphopantetheine [2]. Phosphopantothenic acid has also been demonstrated in the lyase acyl carrier protein [1,3,4]. The finding of one mol each of cysteamine and β-alanine per mol acyl carrier protein (table 1) could, therefore, indicate that 4′-phosphopantetheine would also be the prosthetic group of the lyase acyl carrier protein. However, a striking difference between the fatty acid synthetase and the lyase acyl carrier protein is the presence of one mol adenine in the latter. Therefore, the prosthetic group of the lyase could contain adenine and possibly additional elements of the coenzyme A molecule. This was checked by phosphate and sugar determinations. The results of phosphate determinations are summarized in table 3; they indicate the presence of two mol of organic bound phosphorous per mol protein.

Sugar analyses (table 4) indicate the presence of two mol sugar per mol acyl carrier protein. Partial identification was achieved using a tryptic peptide which contained all of the presumably prosthetic group components. This was subjected to acid hydrolysis (1N HCl; 100°C; 30 min). The hydrolysate was used for paper chromatographic isolation of sugars. Comparison with an authentic sample led to the identification of ribose as one of the two different sugars. The structure of the second sugar residue has not yet been identified. The presence of ribose and adenine was confirmed by the isolation of adenosine, which was characterized by enzymic chromatographic procedures.

Table 3
Phosphate content of acyl carrier protein.

Acetyl acyl carrier protein nmol	Organic phosphorous nmol	Phosphate/acyl carrier protein
6	11.5	1.92
9	17.5	1.95
12	22.7	1.89

Acetyl acyl carrier protein samples were dialyzed for 48 hr against 0.005 M imidazol, pH 6.7, with 1 change of the dialysis medium. Phosphate was determined by the method of Chen et al. [9]. Under our conditions 1 nmol of phosphate corresponded to A_{691} = 0.02. No inorganic phosphate was present.

Table 4
Sugar content of acyl carrier protein.

Sample	Acyl carrier protein nmol	Sugar nmol	Sugar/acyl carrier protein
Acetyl acyl carrier protein	2.2	4.2	1.9
"	4.4	9.0	2.0
"	6.6	12.3	1.9
Carboxymethyl acyl carrier protein	3.7	7.3	2.0
"	4.65	9.9	2.1
"	5.5	12.2	2.2

Sugar analyses were performed by the orcinol method [8] using ribose as a standard; 1 nmol of ribose corresponded to A_{691} = 0.038.

3.4. *Structure of the prosthetic group*

The acyl carrier protein of citrate lyase contains 1 adenine, 1 phosphate and probably 2 sugar residues in addition to the components of phosphopantetheine. It therefore differs from the fatty acid synthetase acyl carrier proteins which have phosphopantetheine alone. The components, which are present in the lyase acyl carrier protein could be bound as sugar, adenylic acid and phosphopantetheine residues to different amino acid residues of the protein. Alternatively all of the components could be linked together in a prosthetic group, which then must be a substituted dephospho CoA or a structural isomer thereof.

To investigate this question a sample of the acyl carrier protein which had been specifically labelled at the cysteamine residue by carboxymethylation with iodo [1-^{14}C]acetate [10] was treated with trypsin. A radioactive peptide was isolated, which contained the adenine, sugar and phosphate residues in the same proportion as that in the intact protein. This peptide was treated with alkali using conditions under which the 4′-phosphopantetheine side chain is cleaved from fatty acid synthetase (0.05 N NaOH; 65°C; 30 min). No trace of 4′-phosphopantetheine could be detected in the hydrolysate; instead, the intact prosthetic group was liberated and could be isolated by paper electrophoresis or thin-layer chromatography.

Another important question concerned the type of binding of the two phosphate residues. Results from acidic and enzymic hydrolyses indicated that pyrophosphate or phosphomonoester linkages were not present in the peptide bound prosthetic group. However, upon alkaline hydrolysis, one of the phosphates was converted to a monoester which could be hydrolysed by alkaline phosphatase. This observation and the ease of alkaline hydrolysis of the prosthetic group from the peptide suggested that the cleavage reaction was analogous to the β-elimination of 4′-phosphopantetheine from a serine residue of fatty acid synthetase [2]. On cleavage of the phosphodiester bond which links 4′-phosphopantetheine to serine, the latter is converted to dehydroalanine which yields pyruvate upon subsequent acidic hydrolysis. A similar experiment with the peptide from the acyl carrier protein of citrate lyase also yielded pyruvate. The results indicate that the different prosthetic groups of fatty acid synthetase and citrate lyase are linked to their respective acyl carrier proteins by a common type of binding, that of a phosphodiester to a serine residue.

The presence of both cysteamine and β-alanine in the prosthetic group of citrate lyase suggested that these were part of a phosphopantetheine residue. This was demonstrated by thin-layer chromatography and paper electrophoresis through comparison with an authentic sample. It had already been established that citrate lyase contains phosphopantothenate [1,3,4]. The harsh condition of alkaline hydrolysis used for liberation of this compound indicates that it had to be 4′-phosphopantothenate rather than the 2′-isomer which is known to be unstable to alkaline hydrolysis [11] and would be undetectable by the analytical procedure used. Therefore, 4′-phosphopantetheine

must be a part of the prosthetic group of the lyase.

The chromatographic and electrophoretic analyses were preceded by cleavage of the isolated prosthetic group with phosphodiesterase (EC 3.1.4.1) which yielded two products, one of which was phosphopantetheine. The second product contained the residual elements of the prosthetic group, i.e. adenine, phosphate and sugar in a ratio of 1:1:2. Since one of these sugar residues was ribose (see above) the adenine containing product is likely to be a substituted adenylic acid. When this compound was hydrolysed (0.1 N HCl; 100°C; 10 min) adenylic acid was liberated as demonstrated by enzymic and chromatographic methods. No adenylic acid was present without acidic hydrolysis. The hydrolysis product yielded ADP on incubation with ATP and myokinase and therefore must be 5′-AMP.

In summary, the results establish the prosthetic group of the acyl carrier protein of citrate lyase as a substituted isomeric dephospho CoA. This is bound to a serine residue of the protein through a phosphodiester linkage of the 5′-phosphate group of adenylic acid. Another phosphodiester linkage binds 4′-phosphopantetheine to the 2′ or 3′ position of ribose. The remaining hydroxyl-group of the ribose is substituted, presumably with a sugar residue.

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