Studies on Adenosine Triphosphate Citrate Lyase of Rat Liver. Binding Site of Citrate*

Fujio Suzuki

ABSTRACT: The binding site of citrate in the citrylated form of ATP citrate lyase was investigated. First, the applicability of Hoare's method was tested using γ-hydroxamyl-*N*-carbobenzoxyglutamylglycinamide as a model substrate. Treatment of this hydroxamic acid derivative with the water-soluble carbodiimide, 1-ethyl-3-dimethylaminopropylcarbodiimide (EDC), at pH 5 was found to result in good conversion into α,γ-diaminobutyric acid by a Lossen rearrangement. Then, using this method the binding site of citrate in the citrylated form of ATP citrate lyase was investigated. The citrylated form of the enzyme was treated with hydroxylamine to yield an enzyme hydroxamate. Lossen rearrangement of the latter with EDC and subsequent hydrolysis yielded α,γ-diaminobutyric acid, indicating the presence of anhydride linkage between citrate and the γ-carboxyl group of a specific glutamyl residue of the enzyme.

From this result it is concluded that the binding site of citrate in the citrylated intermediate of ATP citrate lyase is the γ-carboxyl group of the glutamic acid residue as in the phosphorylated enzyme.

In a series of studies on the reaction mechanism of ATP citrate lyase (EC 4.1.3.8) in this laboratory (Inoue *et al.*, 1967–1969), it was shown that the initial step of the reaction is the activation of the enzyme by ATP to form the phosphorylated enzyme which has a high-energy linkage, followed by conversion of the latter to the citrylated enzyme in the presence of citrate with the concomitant liberation of P_i. The high bond energy between the enzyme and citrate is then retained and transferred to the linkage between the enzyme and acetate, finally being transferred to give rise to acetyl-CoA.

In the previous paper (Suzuki *et al.*, 1969), we presented evidence showing that the binding site of phosphate in the phosphorylated enzyme, the first intermediate, is the γ-carboxyl group of a specific glutamyl residue, after digestion of the complex with Pronase, conversion of the isolated phosphopeptides into the corresponding hydroxamates and subsequent Lossen rearrangement of the latter.

The present paper is on the binding site of citrate in the citrylated enzyme, the second intermediate. The citrylated enzyme is much more labile than the phosphorylated enzyme and the bound citrate is almost completely lost from the enzyme by acid treatment in the cold (Inoue *et al.*, 1968) or during incubation with Pronase for 20 hr at 30° (F. Suzuki, 1969, unpublished data). Therefore, the method used for determination of the binding site of phosphate in the phosphorylated enzyme cannot be applied with the citrylated enzyme. Accordingly another method was used in which the binding site of citrate was trapped directly from the citrylated enzyme without Pronase digestion. Recently, a method for the conversion of hydroxamic acids to the corresponding amines under mild conditions has been developed by Hoare *et al.* (1968). Using this method, treatment of the hydroxamic acid with a water-soluble carbodiimide at pH 5 was found to result in quantitative conversion to the amine by a Lossen rearrangement at room temperature. In this work, Hoare's method was successfully applied to the determination of the binding site of citrate in the citrylated enzyme.

Experimental Section

Assays. Hydroxamic acids were assayed by the method of Lipmann and Tuttle (1945). Amino acids were determined by the procedure of Troll and Cannan (1953).

High-Voltage Paper Electrophoresis. High-voltage electrophoresis for identification of amino acids was carried out on Toyo Roshi No. 51A paper (60 cm) moistened with pyridine–acetate buffer, pH 3.5 (pyridine–acetic acid–water, 2.5:30:1800, v/v) for 30 min at 3000 V in a hexane tank cooled with ice water.

Paper Chromatography. In the model experiment, paper chromatography was carried out for removal of hydroxylamine using 77% ethanol as solvent. The R_F values for hydroxylamine and DAB[1] were 0.86 and 0.20, respectively.

For determination of the binding site of citrate, the eluate from Dowex 50 column was applied to Toyo Roshi No. 51A paper, and subjected to descending chromatography using a solvent system of 1-butanol–pyridine–water (1:2:2, v/v). After development for 20 hr, the markers, histidine, DAB, and DAP, were located by spraying the paper with 0.1% ninhydrin in ethanol. The R_F values of histidine, DAB, and DAP were 0.55, 0.19, and 0.16, respectively. Histidine, which interferes with the subsequent analysis of DAB and DAP, can be satisfactorily eliminated by this procedure.

Amino Acid Analysis. The sample was applied on a 45-cm column of Chromosorb No. 3105 and elution was effected with 0.35 M sodium citrate buffer (pH 5.28) at 50°. The respective positions of the standards DAB and DAP on this column were established. This analysis was carried out by Dr. M. Ebata of the Institute of The Shionogi Pharmaceutical Co., Ltd. (2-47, Sagisukami, Fukushima-Ku, Osaka).

Preparation of Enzyme. Purified ATP citrate lyase was prepared from the livers of rats after induction with a high sucrose diet, as reported previously (Inoue *et al.*, 1966).

Preparation of Citrylated Enzyme and Enzyme Hydroxamate. Phosphorylated enzyme and citrylated enzyme were prepared as outlined in the previous paper (Inoue *et al.*, 1968). The

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[1] Abbreviations used in this work are: DAB, α,γ-diaminobutyric acid; DAP, α,β-diaminopropionic acid; EDC, 1-ethyl-3-dimethylaminopropylcarbodiimide.

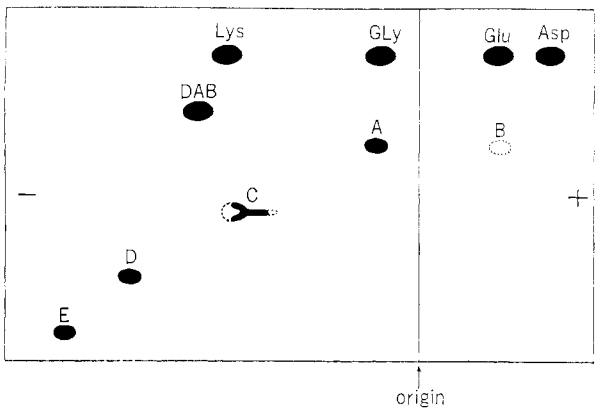

FIGURE 1: Paper electrophoregrams of fractions from Dowex 50 column. Conditions were as described in the text. (A and B) Fractions eluted with 2 N pyridine, (C) fraction eluted with 2 N ammonia, (D) fraction eluted with 4 N ammonia, and (E) fraction eluted with 6 N ammonia.

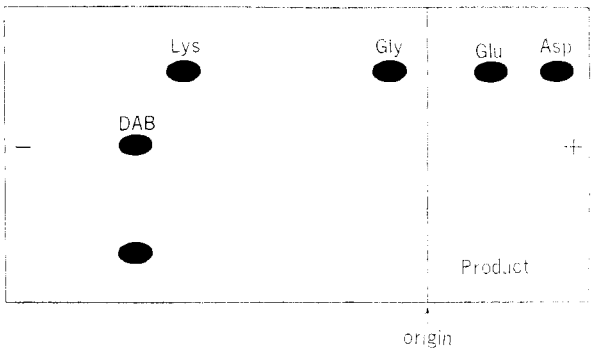

FIGURE 2: Identification of the reaction product by electrophoresis. Conditions were as described in the text.

reaction mixture contained (in μmoles) Tris-HCl (pH 8.0, 250), dithiothreitol (50), $MgCl_2$ (50), ATP (30), and enzyme (0.2, 100 mg) in a total volume of 6 ml. After incubation for 10 min at 37°, 400 μmoles of potassium citrate was added and the incubation was continued for another 2 min. Then the reaction mixture was treated with 0.2 M hydroxylamine for 15 min at 37°, as described in the previous paper (Suzuki *et al.*, 1969). After treatment, the mixture was chilled and the protein portion was isolated by gel filtration on a Sephadex G-50 column (2.2 × 84 cm), equilibrated with 0.05 M Tris-HCl (pH 8.0) containing 0.01 M mercaptoethanol and 0.001 M $MgCl_2$. The protein portion was incubated with 6 μmoles of EDC for 1 hr at 25°, maintaining the pH at 5 by titration with 0.05 N HCl during the reaction. After incubation, the reaction mixture was lyophilized.

Materials. Samples of aspartyl-β-ethyl ester, γ-benzyl-*N*-carbobenzoxyglutamylglycinamide, and EDC were obtained by courtesy of Dr. S. Sakakibara, the Institute for Protein Research of this University. Authentic DAB and DAP were kindly donated by Professor Y. Izumi of the Institute for Protein Research, Osaka University. Aspartyl-β-hydroxamic acid and γ-hydroxamyl-*N*-carbobenzoxyglutamylglycinamide were prepared by the method of Hauser and Renfrow (1943). Other materials used were available commercially.

Results

Test of Hoare's Method. First the applicability of Hoare's method was tested using a model substrate, aspartyl-β-hydroxamic acid. EDC was used throughout as a water-soluble carbodiimide instead of the 1-benzyl derivative. An aqueous solution (10 ml) of aspartyl-β-hydroxamic acid (0.15 mmole) was incubated with 1.5 mmoles of EDC for 1 hr at 25°, maintaining the mixture at pH 5.0 by titration with 0.05 N HCl during the reaction. Then the reaction mixture was reduced to a small volume under reduced pressure at room temperature with the aid of a flash evaporator. An aliquot of the concentrated mixture was subjected to high-voltage paper electrophoresis. However, unexpectedly no DAP was detected on the electrophoregram. The reason for this is unknown but it is possible that, the presence of EDC favors the polymerization of aspartic acid rather than the reaction of the hydroxamyl group, since both the N- and C-terminal groups of the substrate are free.

Next a compound in which the amino and carboxyl groups are both protected, γ-hydroxamyl-*N*-carbobenzoxyglutamylglycinamide, was used as a model substrate. An aqueous solution (15 ml) of this compound (0.13 mmole) was incubated with 3 mmoles of EDC as described above. After incubation, the mixture was evaporated to a small volume under reduced pressure at room temperature with the aid of a rotary evaporator. The concentrated mixture was then transferred to a test tube and dried *in vacuo*. The residue was hydrolyzed by heating it in a sealed, evacuated tube for 5 hr at 110° with 4 ml of glass-distilled 6 N HCl. The acid hydrolysate was evaporated to dryness under reduced pressure and the residue was dissolved in water. The resulting solution was decolorized by treatment with charcoal and filtration. The filtrate was chromatographed on a column of Dowex 50 (H^+ form) (1.8 × 15 cm), eluting successively with 2 N pyridine and 2, 4, and 6 N aqueous ammonia. The products in these eluates were then subjected to high-voltage paper electrophoresis and located by spraying the paper with ninhydrin. The results are given in Figure 1. Among the ninhydrin-reactive products, spots A and B, which were both eluted with 2 N pyridine, were located in the same positions as glycine and glutamic acid, respectively. Spot C, eluted with 2 N ammonia, moved very close to, but slightly slower to the anode than authentic DAB and it migrated together with a diffuse yellow spot, which is thought to be hydroxylamine, a degradation product of the substrate. Two other spots (D and E), which were more basic and gave a dark blue color with ninhydrin, were detected in the eluates with 4 N and 6 N ammonia. These spots were not analyzed further, but their strong basic behaviors suggest that they are compounds related to urea derived from EDC.

To characterize spot C further, the eluate with 2 N ammonia was concentrated *in vacuo* and subjected to ascending paper chromatography using 77% ethanol as solvent to remove hydroxylamine, which interfered with the material during electrophoresis. After development for 16 hr at room temperature, the ninhydrin-positive area was cut out and eluted with distilled water. An aliquot of the eluate was resubjected to high-voltage paper electrophoresis. As shown in Figure 2, the product was located at exactly the same position as authentic DAB on the electrophoregram. To calculate the total recovery of the product, DAB, a fixed quantity of the eluate was subjected to preparative paper electrophoresis and the isolated DAB was measured by the method of Troll and Cannan (1953). The yield of DAB was estimated to be about 60% on the basis of the amount of the substrate, γ-hydroxamyl-*N*-carbobenzoxyglutamylglycinamide, used.

Binding Site of Citrate in the Citrylated Enzyme. The above

experiments with a model substrate, suggest that the method of Hoare *et al.* (1968) is suitable for the present purpose. Thus, this method was used to determine the amino acid residue binding citrate in the citrylated enzyme, namely, to test whether the γ-carboxyl group of glutamic acid residue is responsible for the binding of citrate as in the phosphorylated enzyme.

A purified sample of ATP citrate lyase (960 mg) was incubated with ATP in the presence of Mg^{2+} to obtain phosphorylated enzyme. Each run was carried out with about 100 mg of enzyme, as described in the Experimental Section. After reaction for 10 min, a large excess of potassium citrate was added to the mixture and incubation was continued for an additional 2 min. This treatment converted the phosphorylated enzyme almost completely into the citrylated enzyme with concomitant release of P_i (Inoue *et al.*, 1968). Then, the mixture was further incubated with hydroxylamine and the protein portion was isolated by column chromatography on Sephadex G-50. Free hydroxylamine, which interferes with the separation of DAB in subsequent steps, was removed by this procedure. No hydroxamate derivative was detected in the nonprotein fraction of these column eluates. The isolated protein was then treated with EDC. Then the mixture was lyophilized and hydrolyzed by heating it with 6 N HCl in a sealed, evacuated tube for 20 hr at 110°. The acid hydrolysate was evaporated to dryness under reduced pressure and the residue was dissolved in water. The aqueous solution was decolorized by treatment with charcoal and filtered. The filtrate was chromatographed on a column of Dowex 50 (H^+ form). Elution was carried out in the same way as in the model experiment. The fractions eluted with 2 N aqueous ammonia were combined and concentrated to dryness *in vacuo*. The dried material was then subjected to preparative paper electrophoresis to isolate basic amino acids. The area corresponding to the positions of DAB and DAP was cut out and eluted with water. After concentration, the eluate was subjected to preparative paper chromatography as described in Experimental Section. The area corresponding to the positions of DAB and DAP on the paper chromatogram was cut out and eluted with water. As a control, a mixture of enzyme and citrate was treated in exactly the same way as the citrylated enzyme. The final eluates, from the citrylated enzyme and the control, were subjected to amino acid analysis. As shown in Figure 3, DAB is formed from the hydroxylamine-treated citrylated enzyme after reaction with EDC and acid hydrolysis, whereas it is not formed from the mixture of enzyme and citrate after the same treatments. No detectable amount of DAP is formed from either the citrylated enzyme or the control. The recovery of the product, DAB, is about 7% on the basis of the amount of enzyme used. However, the low recovery is probably due to lability of the citrylated enzyme intermediate, some of which is degraded during incubation. In accordance with this, it was found that the molar ratio of citrate to enzyme in the complex is low, ranging from 0.1 to 0.24, after isolation through a column of Sephadex G-50 (Inoue *et al.*, 1968).

From these results, it is concluded that the binding site of citrate in the citrylated intermediate of ATP citrate lyase is the γ-carboxyl group of glutamic acid residue as in the phosphorylated intermediate. Activation of this group by anhydride linkage to the substrate, citrate, is required for formation of the hydroxamic acid and subsequent isolation of DAB.

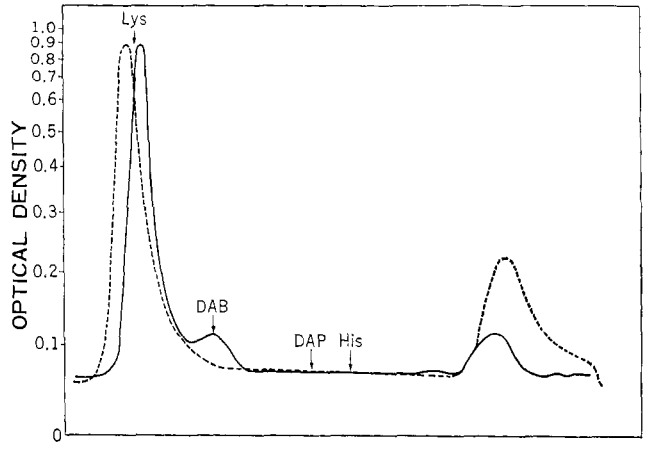

FIGURE 3: Identification of α,γ-diaminobutyric acid. Experimental conditions and procedures for amino acid analysis were as described in the text. (———) Sample obtained from the citrylated enzyme. (--------) Control sample.

Discussion

This work shows that the citrylated site of ATP citrate lyase is a glutamyl residue as in the phosphorylated enzyme. This finding strongly supports the view that the high bond energy derived from ATP is first trapped in the γ-carboxyl group of the glutamyl residue of the enzyme to form enzyme–glutamyl phosphate. Then this bound phosphate is replaced by citrate to form an acid anhydride linkage between the enzyme and citrate at the same site, leading to the formation of enzyme–glutamyl–citrate as the second reactive intermediate. Though not directly examined, the present results also suggest that the binding site of acetate in the acetylated enzyme, the third reactive intermediate, is the same as the citrylated site, since the acetylated enzyme is derived directly from the citrylated enzyme by eliminating the oxaloacetate moiety from the bound citrate (Inoue *et al.*, 1969). Thus, it follows that, during the reaction of ATP citrate lyase, the high bond energy from ATP is retained and moves in order through the linkages between the enzyme and phosphate, enzyme and citrate, and enzyme and acetate at the γ-carboxyl group of the glutamyl residue of the enzyme, finally being transferred to give rise to acetyl-CoA.

The evidence presented here also indicates that the γ-carboxyl group of the glutamyl residue is solely attacked by hydroxylamine, since DAB was obtained after Lossen rearrangement and subsequent hydrolysis of the citrylated enzyme, and no hydroxamate derivative was detected in the nonprotein fraction of the Sephadex G-50 column eluates. In this connection, it was shown that acetohydroxamate from the citrylated enzyme and hydroxylamine was formed only in the presence of CoA (H. Inoue *et al.*, unpublished data).

In the previous report, we showed that the binding site of phosphate in the phosphorylated intermediate of ATP citrate lyase is γ-carboxyl group of the glutamic acid residue (Suzuki *et al.*, 1969). In a preliminary report, Cottam and Srere (1969) confirmed our results on the formation of phosphorylated enzyme. Then, they isolated [^{32}P]phosphohistidine from an alkaline hydrolysate of enzyme labeled with ^{32}P and suggested that the phosphorylated site on the enzyme may be an imidazole residue. In their experiments, however, the hydrolysis procedure used was rather drastic, namely, the ^{32}P-labeled enzyme was treated with 3 N NaOH for 15 hr at 100° and then subjected to column chromatography for identification of the products. This drastic treatment may result in formation of artifacts by migration of the bound phosphate during hydrol-

ysis, as pointed out by Wålinder (1968) and by us (Suzuki *et al.*, 1969). The demonstration that the binding site of citrate in the citrylated enzyme is the γ-carboxyl group of the glutamic acid residue provides additional evidence for the participation of the glutamic acid residue in the binding of phosphate in the phosphorylated enzyme.

It is conceivable that the formation of an anhydride linkage between citrate and the γ-carboxyl group of the glutamyl residue of the enzyme results in electron withdrawal into the enzyme and in electron deficiency in the bond between C-2 and C-3 of the bound citrate molecule. This would enhance the breaking of the C–C bond between C-2 and C-3 of the citrate and facilitate the slitting off of the oxaloacetate moiety by the action of CoA.

In this connection, particular attention should be paid to the similarities in the reaction mechanism of carbon–carbon lyases and carbon–nitrogen lyases. The reaction mechanism of enzymes of the latter type, such as L-histidine ammonia-lyase (EC 4.3.1.3) and L-phenylalanine ammonia-lyase (EC 4.3.1.5), has been investigated extensively in recent years (Givot *et al.*, 1969; Havir and Hanson, 1968). In these enzymes, a reactive carbonyl group on the enzyme molecule is considered to take part in the catalytic process. The breaking of the N–C linkage is facilitated by formation of a carbonylamine intermediate. This intermediate then gives rise to either urocanate or cinnamate and to a carbonyl ammonia. According to this mechanism, the amino group of the substrate combines with the carbonyl group of the enzyme to form a protonated carbinolamine or possible a protonated Schiff base. Thus, the interaction between the carbonyl group and amino group results in the formation of a group which is more easily split off and facilitates the elimination of ammonia. Recently, Hodgins and Abeles (1969) reported that D-proline reductase (EC 1.4.1.6), which is not a carbon–nitrogen lyase, but also catalyzes the breaking of the N–C bond, contains pyruvate covalently bound to enzyme protein and that this carbonyl group of pyruvate may be involved in the N–C cleavage. Therefore, in all the above cases, the interaction of substrate with enzyme to form a covalently bound enzyme–substrate complex enhances the breaking of the carbon–carbon bond or carbon–nitrogen bond of the substrate.

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