Identification of lysine-238 of *Escherichia coli* biotin carboxylase as an ATP-binding residue

Yasuaki Kazuta*, Eiko Tokunaga, Eiji Aramaki, Hiroki Kondo

Department of Biochemical Engineering and Science, Kyushu Institute of Technology, Iizuka 820, Japan

Received 9 March 1998; revised version received 16 April 1998

Abstract ***Escherichia coli*** **biotin carboxylase was affinity labeled with adenosine diphosphopyridoxal to identify its ATP binding site. Lysyl endopeptidase digestion of the modified protein, followed by high performance liquid chromatography separation and amino acid sequencing allowed to identify lysine-238 to be the site of modification. Site-directed mutagenesis of this residue into alanine, arginine or glutamine resulted in mutants with much decreased activity. Lysine-238 seems to interact with the γ-phosphate group of ATP but is not involved in catalysis.**

Key words: Biotin carboxylase; Acetyl-coenzyme A carboxylase; Affinity labeling; Adenosine diphosphopyridoxal; Adenosine triphosphate-binding site; Site-directed mutagenesis

1. Introduction

Acetyl-CoA carboxylase (ACC) is the rate-determining enzyme in fatty acid synthesis. ACC from *Escherichia coli* and other bacteria is composed of the following three subunits: biotin carboxylase (BC) which mediates the carboxylation of enzyme-bound biotin, carboxyl transferase (CT) which mediates transfer of the carboxyl group from carboxybiotin to acetyl-CoA and the biotin-carrying protein [1,2]. On the basis of the deduced amino acid sequence of *E. coli* BC, the glycine-rich region Gly-Gly-Gly-Gly-Arg-Gly at amino acid numbers 163–168 was presumed to be the ATP-binding site of this enzyme [3]. But according to the recently reported three-dimensional structure of *E. coli* BC, the glycine-rich region is located in the 'B domain', away from the center of the putative active site cavity [4]. Therefore, precise location of ATP-binding sites remains to be solved. To identify these sites, we used an affinity labeling technique. The labeling agent adopted was adenosine diphosphopyridoxal (AP_2-PL), which is expected and has been proven to modify lysine residue(s) located at the ATP-binding site of enzymes [5]. The data shown below reveal that this reagent modified exclusively Lys-238 located in the putative active site cavity of BC. Site-directed mutagenesis of Lys-238 shows that this residue is involved in the binding of ATP with electrostatic interaction.

*Corresponding author. Fax: (81) (948) 297801.
E-mail: kazuta@bse.kyutech.ac.jp

Abbreviations: ACC, acetyl-CoA carboxylase; BC, biotin carboxylase; CPS, carbamoyl-phosphate synthetase; AP_2-PL, adenosine diphosphopyridoxal; IPTG, isopropyl-β-D-thiogalactopyranoside; HEPES, *N*-2-hydroxyethylpiperazine-*N*′-2-ethanesulfonic acid

2. Materials and methods

2.1. Materials

Taq DNA polymerase, plasmid pTV119N and restriction enzymes were purchased from Takara Shuzou, Kyoto. Plasmid pTrc99A and *E. coli* strain JM109 were obtained from Pharmacia. Plasmid pBluescript II SK+ was from Stratagene. Biotin and lysyl endopeptidase were obtained from Wako, Osaka. Oligonucleotides were synthesized with an Applied Biosystems DNA synthesizer model 391. AP_2-PL was synthesized as described previously [6]. High performance liquid chromatography was run on a Hitachi chromatograph. Reversed phase C4 and C18 columns were purchased from Nacalai Tesque, Kyoto. Fluorescence chromatograms were obtained on a Hitachi spectrophotometer F-1050. The fluorescence intensity of a pyridoxamine derivative prepared by treating a mixture of 100 μM AP_2-PL and 1 M tris(hydroxymethyl)aminomethane with 1 mM sodium borohydride was taken as the standard for the BC-bound fluorescence derived from AP_2-PL. Amino acid sequence was determined on an Applied Biosystems sequenator 491A.

2.2. Construction of an expression plasmid for BC

An over-expression plasmid for *E. coli* BC was prepared by ligating its gene (1347 bp) with plasmid pTrc99A. The details of this construction will be reported elsewhere. Briefly, the entire coding region of *E. coli* BC and its downstream region were divided into four segments (S1–S4 from the 5′ terminus). Their sizes were the following: S1, 396 bp; S2, 426 bp; S3, 205 bp; and S4, 337 bp. Each segment was endowed a restriction enzyme site at both ends by minimal mutation of bases in such a way that no amino acid substitution is produced with an exception of the second residue from the amino terminus. Individual segments were amplified separately by the polymerase chain reaction in a 100-μl solution containing 10 mM Tris-HCl (pH 8.9), 25 mM $MgCl_2$, 80 mM KCl, 0.2 mM each of deoxynucleotides (dATP, dGTP, dCTP and dTTP), 0.2 μM each of a combination of appropriate primers, 0.5 ng of pAK-1, *E. coli* genomic DNA clone containing the entire BC coding region [3], and 5 units of *Taq* polymerase with a program consisting of 94°C for 3 min, 50°C for 2 min and 72°C for 3 min in the first cycle and 94°C for 1 min, 50°C for 2 min and 72°C for 3 min in 2–35 cycles in a thermal cyclic reactor (Astec, Fukuoka, PC-700). *E. coli* DNAs thus amplified were digested with appropriate restriction enzymes and ligated into plasmid pBluescript II SK+ or pTV119N to determine nucleotide sequence [7]. It was confirmed that no undesired mutation had taken place on the amplified DNAs. The insert DNAs were recloned into pTrc99A to complete an expression plasmid for BC, pTBC. Because of the engineering of its sequence, pTBC generated *E. coli* BC with Leu-2 replaced with Val, which was confirmed by amino acid sequencing.

2.3. Site-directed mutagenesis of Lys-238 of BC

Lys-238 was in the S2 (*Sma*I/*Kpn*I) segment of pTBC. Hence, mutated forms of S2 need to be prepared to construct Lys-238 mutants. This was carried out by the polymerase chain reaction with the oligonucleotides listed in Fig. 1 as primers. Primer A1 corresponds to nucleotides 337–369 in the coding sequence of BC. Primer A2 was designed to cover nucleotides 715–747. Primer A3 was designed to cover nucleotides 847–871 in the complementary sequence. Primers A4(Ala), A4(Gln) and A4(Arg) were designed to convert Lys-238 to Ala, Gln and Arg, respectively and were to cover nucleotides 688–720 in the complementary sequence. The PCR reaction was performed with 0.2 μM each of primers A1 and A4(Ala), A4(Gln) or A4(Arg) and 0.5 ng of pTBC under the conditions described above to give S2-1. Likewise, S3-1 was prepared with primers A2 and A3 and 0.5 ng of

PII S0014-5793(98)00472-4

```
A1:       5'-ATGGGCGACAAAGTATCCGCAATCGCGGCGATG-3'
A2:       5'-GTGGTCGAAGAAGCGCCAGCACCGGGCATTACC-3'
A3:       5'-GTAGCCCGCGTAGATATGAGACTCCCAACGTAC-3'
A4(Ala): 5'-TTCGACCACTTGCTGGTGGCGGCGTTGGCTGGA-3'
A4(Glu): 5'-TTCGACCACTGCCTGGTGGCGGCGTTGGCTGGA-3'
A4(Arg): 5'-TTCGACCACGCGCTGGTGGCGGCGTTGGCTGGA-3'
```

Fig. 1. Oligonucleotides used for site-directed mutagenesis of Lys-238 of *E. coli* BC. The underlines represent the mutated site.

pTBC. The PCR products were electrophoresed in agarose gel and recovered with a Geneclean II DNA purification kit. A second round of PCR was then performed analogously with 0.5 ng each of the PCR products S2-1 and S3-1 as primers and template with a program consisting of 94°C for 3 min, 40°C for 2 min and 60°C for 3 min in the first cycle and 94°C for 1 min, 40°C for 2 min and 62°C for 3 min in 2–35 cycles. This PCR produced a ~1000-bp DNA fragment. *Sma*I (nucleotides 792–797) and *Kpn*I (nucleotides 817–822) digestion yielded a 426-bp DNA fragment (S2m). It was purified by agarose gel electrophoresis, ligated into pBluescript II SK+ and DNA sequence was determined. Thus, mutants replacing Lys-238 with Ala, Gln or Arg, denoted as K238A, K238Q and K238R, respectively, were obtained.

2.4. Determinatin of enzyme activity and protein concentration

Over-expressed BC was purified in a way analogous to that for native BC [2] with some modifications. Enzyme activity was measured by the spectrophotometric method of Tipton and Cleland [8] using free biotin as a carboxyl acceptor. Protein concentration was determined either by the micro-Biuret method [9] or by the Bradford method [10]. The K_m values of the recombinant enzyme were determined from double-reciprocal plots of the BC activity against substrate concentration.

3. Results

3.1. Properties of over-expressed BC

To obtain mutants of biotin carboxylase (BC), an over-expression system was established based on a plasmid (pTBC) which is capable of expressing *E. coli* BC with Leu-2 replaced with Val. *E. coli* JM109 cells carrying pTBC over-expressed a protein with an apparent molecular weight of 51 000 in the presence of IPTG. This value is consistent with the protomer molecular weight of *E. coli* BC [1,2]. BC was over-produced about 2000-fold over endogenous under the present conditions. The over-expressed BC was purified to higher than 95% homogeneity as assessed by sodium dodecylsulfate-polyacrylamide gel electrophoresis (Fig. 2). About 30 mg of BC were obtained from 2 l of cell culture. The specific activity of the purified BC, 1.1 units/mg, was comparable to that of the native [8]. The K_m values for the three substrates, biotin, bicarbonate and ATP (Table 1), were also comparable to those given in the literature [11]. This preparation was used in the following modification experiments.

The mutant enzymes K238A, K238Q and K238R were isolated in yields of 5, 5 and 30 mg from a 2-l cell culture, respectively. The lower yields of K238A and K238Q were due to their vulnerability to proteolysis: they underwent limited proteolysis to give a smaller molecular weight protein

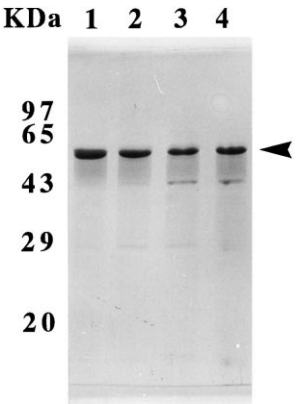

Fig. 2. SDS electrophoresis of 0.8 μg each of wild-type and mutated forms of BC on a 12.5% polyacrylamide gel. Lane 1: wild-type; lane 2: K238A; lane 3: K238Q; lane 4: K238R. The arrowhead indicates the band of BC.

(35 or 27 kDa) during purification. Once isolated in pure form, they were as stable as the wild type.

3.2. Affinity labeling of BC

BC (50 μM) was allowed to react with 100 μM AP_2-PL at 4°C for various periods of time. Following reduction with 1 mM sodium borohydride, the residual enzyme activity was determined. As shown in Fig. 3, the enzyme activity decreased with the time of incubation with the reagent. Unlike AP_2-PL, PLP was hardly capable of inactivating BC under the same reaction conditions. ATP, ADP, inorganic phosphate and bicarbonate protected BC from inactivation by AP_2-PL to varying degrees, ATP giving the greatest protection (Fig. 4).

To determine the stoichiometry of inactivation by AP_2-PL, correlation of the residual enzyme activity with the fluorescence intensity of the sample was studied. BC was modified by AP_2-PL in a similar manner. The inactivation mixtures were applied to a gel filtration column [12]. The fluorescence of the pyridoxyl lysine moiety, protein concentration, enzyme activity of flow-through fractions were measured. As shown in Fig. 5, the fluorescence intensity increased linearly with a decrease in enzyme activity. Extrapolation of the line to zero enzyme activity gave a stoichiometry of 1.02 mol of reagent bound per mol of enzyme.

Following dialysis, the inactivated enzyme (12.5 mg) was digested with lysyl endopeptidase (w/w, 1:15) at 37°C for 24 h. The resulting peptide mixture was separated by high performance liquid chromatography on a C4 column. As shown in Fig. 6, a virtually single fluorescent peptide was observed, which accounted for at least 70% of the total fluorescence. This peptide was rechromatographed on a C18 column to give a nearly homogeneous peptide. The amino acid sequence of this peptide was determined to be Gln-Arg-Arg-His-Gln-Xaa. At the sixth cycle of Edman degradation an

Table 1
Kinetic parameters for wild-type and mutant BCs

Enzyme	V_{max} (units/mg)	K_m (mM)		
		ATP	Bicarbonate	Biotin
Wild-type	1.67	0.17	1.8	160
K238A	0.66	21	3.7	180
K238Q	0.44	8.6	3.9	140
K238R	0.32	13	6.8	180

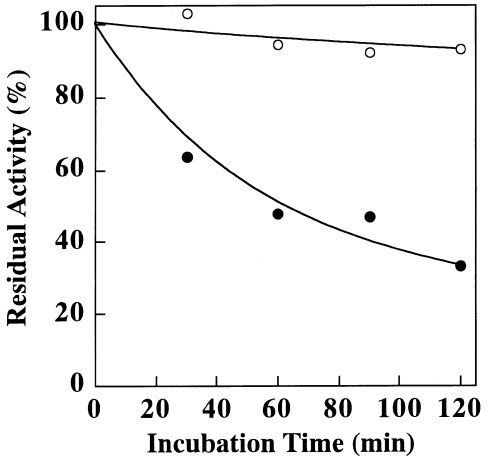

Fig. 3. Time course of inactivation of BC by AP_2-PL or PLP. A inactivation mixture (100 μl) containing 50 μM BC, 25 mM HEPES (pH 8.0), 5 mM $MgCl_2$, 20% glycerol and 100 μM AP_2-PL (●) or PLP (○) was incubated at 4°C. At the indicated time, the mixture was reduced with 1 mM sodium borohydride. The residual activity was measured.

unknown amino acid (Xaa) was released and after that identification of any amino acid was no more possible. This sequence up to the fifth is completely consistent with amino acid numbers 233–237 of *E. coli* BC. Since the amino acid at 238 is Lys, the data suggest strongly that it was Lys-238 which was modified by AP_2-PL. Of the 30 lysine residues of *E. coli* BC virtually a single lysine was modified with high specificity.

3.3. Properties of Lys-238 mutants of BC

To assess the role of Lys-238, this residue was converted to Ala, Arg or Gln by site-directed mutagenesis. Kinetic studies on the biotin-dependent ATP degradation of these mutants K238A, K238R and K238Q were carried out. All the mutants showed more or less activity; K238A was the most active and rather surprisingly K238R was the least active (Table 1). Their

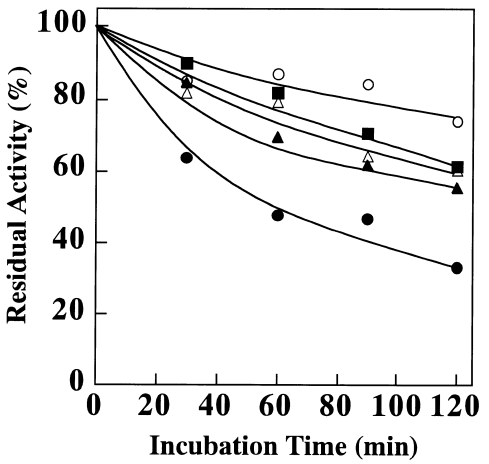

Fig. 4. Effect of substrates on the inactivation of BC by AP_2-PL. The inactivation mixture (100 μl) containing 50 μM BC, 25 mM HEPES (pH 8.0), 5 mM $MgCl_2$, 20% glycerol and 100 μM AP_2-PL (●) plus 1 mM ATP (○), 5 mM bicarbonate (▲), 5 mM ADP (△), or 5 mM inorganic phosphate (■) was incubated at 4°C. At the indicated time, the mixture was reduced with 1 mM sodium borohydride and the residual activity was measured.

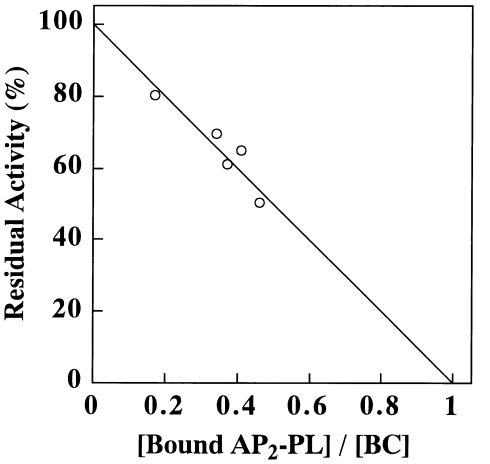

Fig. 5. Relationship between inactivation of BC by AP_2-PL and the amount of label incorporated into the monomer of the dimeric enzyme. The enzyme concentration was determined by the micro-Buret method [8].

K_m values for ATP were 50–120 times higher than that of wild-type, while the K_m values of the mutants for bicarbonate increased 2–4 times that of the wild type. The K_m value for biotin was not affected much by the substitution. These data indicate that the binding of ATP was impaired most by the substitution of Lys-238.

4. Discussion

As shown above, Lys-238 of *E. coli* BC was specifically modified by AP_2-PL and mutation of this residue resulted in a marked decrease in its ATP binding ability, strongly sug-

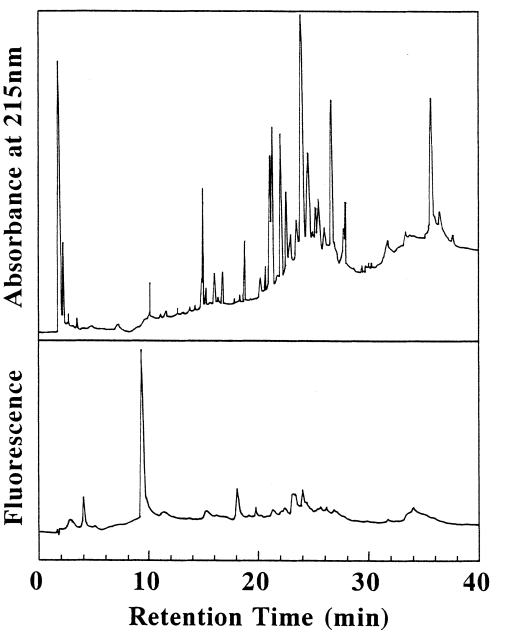

Fig. 6. High-performance liquid chromatography of the AP_2-PL-labeled peptide of BC. The labeled enzyme was digested with lysyl endopeptidase and applied to a C4 column. The peptides were eluted by linear gradient of 0.1% trifluoroacetic acid to 0.095% trifluoroacetic acid containing 60% acetonitrile over 40 min at flow rate 1 ml/min. Absorbance at 215 nm (upper panel) and fluorescence at 390 nm excited at 325 nm (lower panel) were monitored.

gesting that Lys-238 participates in the binding of ATP. This finding is consistent with the X-ray diffraction data on BC, which revealed that Lys-238 is located in the putative active site cavity [4]. Also present in this cavity is a universally conserved sequence, Glu-X-Asn-X-Arg, in amino acid numbers 288–292. Site-directed mutagenesis of these three residues, Glu, Asn and Arg, resulted in total loss of enzymic activity in some cases (unpublished observation in this laboratory), suggesting that they participate directly in the catalysis of this enzyme. Lys-238 is in close proximity to these residues and if the γ-phosphate of ATP is in contact with this residue, the phosphate could be readily transferred by the action of the catalytic machinery of the enzyme. Furthermore, Lys-238 is conserved in all of the biotin-depending enzymes which have been sequenced to date, suggesting a common role in all of them.

Biotin-dependent carboxylases including *E. coli* BC share a Gly-rich sequence in the form of Gly-Gly-Gly-Gly-Arg-Gly or Gly-Gly-Gly-Gly-Lys-Gly [13], which have been supposed to be their ATP-binding site [3,14]. This notion was based on a sequence analogy to the well-established nucleotide-binding sequence Gly-X-X-X-X-Gly-Lys [15,16], where the Lys undergoes electrostatic interaction with ATP. The Gly-rich sequence of BC is located in the B domain, far away from the putative active site composed of such residues as Glu-288 and Arg-292 (see above). When ATP is anchored at Lys-238 electrostatically no part of the nucleotide seems to interact with the Gly-rich region, unless the protein undergoes a drastic conformational change. Some conformational change seems to be probable, since the Gly-rich region of the B domain was reported to be flexible [4], but a change as large as to enable the Gly-rich sequence to come into contact with the cavity-bound ATP is highly unlikely. Hence, we have to conclude that the Gly-rich sequence of BC and presumably of related enzymes does not participate in ATP binding.

Recently, it was found that the three-dimensional structure of BC is similar to those of some ADP-forming ligases [17,18]. Carbamoyl-phosphate synthetase (CPS) is such an example, shares conserved primary sequences with BC and is believed to have evolved from a common ancestor [13]. The B domain of CPS, carrying the conserved Gly-rich sequence, was assumed to undergo a conformational change upon nucleotide binding [18]. Furthermore, mutation of the Gly-rich sequence of CPS resulted in a significant decrease in its activity [19]. The Gly-rich sequence of glutathione synthetase, another ADP-forming ligase, is supposed to serve as a lid for the active site cavity, thereby holding enzyme-bound substrates in correct orientation or shielding the active site from water [20]. By reference to these observations, it may be reasonable to assume that the Gly-rich region of BC plays a similar important role in the functioning of BC. A role as a lid for the active site is an attractive possibility, since the B domain of BC is located near the entrance to the cavity, though its orientation relative to the main body is different from that of the other ADP-forming ligases [17,18]. This hypothesis and other possibilities including a conformational change of the B domain await experimental verification.

References

[1] Alberts, A.W., Nervi, A.M. and Vagelos, P.R. (1969) Proc. Natl. Acad. Sci. USA 63, 1319–1326.
[2] Guchhait, R.B., Polakis, S.E., Dimroth, P., Stoll, E., Moss, J. and Lane, M.D. (1974) J. Biol. Chem. 249, 6633–6645.
[3] Kondo, H., Shiratsuchi, K., Yoshimoto, T., Masuda, T., Kitazono, A., Tsuru, D., Anai, M., Sekiguchi, M. and Tanabe, T. (1991) Proc. Natl. Acad. Sci. USA 88, 9730–9733.
[4] Waldrop, G.L., Rayment, I. and Holden, H.M. (1994) Biochemistry 33, 10249–10256.
[5] Colman, R.F. (1990) in: The Enzymes (Sigman, D.S. and Boyer, P.D., Eds.) Vol. 19, pp. 283–321, Academic Press, New York.
[6] Tagaya, M. and Fukui, T. (1986) Biochemistry 25, 2958–2964.
[7] Sambrook, J., Fritsch, E.F. and Maniatis, T. (1989) in: Molecular Cloning: A Laboratory Manual (Second Edition), Cold Spring Harbor Laboratory Press, Cold Spring Harbor, New York, NY.
[8] Tipton, P.A. and Cleland, W.W. (1988) Biochemistry 27, 4317–4325.
[9] Munkers, K.D. and Richards, F.M. (1965) Arch. Biochem. Biophys. 109, 466–478.
[10] Bradford, M.M. (1976) Anal. Biochem. 72, 248–254.
[11] Polakis, S.E., Guchhait, R.B., Zwergel, E.E., Lane, M.D. and Cooper, T.G. (1974) J. Biol. Chem. 249, 6657–6667.
[12] Penefsky, H.S. (1977) J. Biol. Chem. 252, 2891–2899.
[13] Toh, H., Kondo, H. and Tanabe, T. (1993) Eur. J. Biochem. 215, 687–696.
[14] Takai, T., Yokoyama, C., Wada, K. and Tanabe, T. (1988) J. Biol. Chem. 263, 2651–2657.
[15] Walker, J.E., Saraste, M., Runswick, M.J. and Gay, N.J. (1982) EMBO J. 1, 945–951.
[16] Moller, W. and Amons, M. (1985) FEBS Lett. 186, 1–7.
[17] Artymiuk, P.J., Poirrette, A.R., Rice, D.W. and Willett, P. (1996) Nat. Struct. Biol. 3, 128–132.
[18] Thoden, J.B., Holden, H.M., Wesenberg, G., Raushel, F.M. and Rayment, I. (1987) Biochemistry 36, 6305–6316.
[19] Post, L.E., Post, D.J. and Raushel, F.M. (1989) J. Biol. Chem. 265, 7742–7747.
[20] Hibi, T., Nishioka, T., Kato, H., Tanizawa, K., Fukui, T., Katsube, Y. and Oda, J. (1996) Nat. Struct. Biol. 3, 16–18.