Detection of ligand-induced perturbations affecting the biotinyl group of mammalian acetyl-coenzyme A carboxylase by using biotin-binding antibodies

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Biotin-binding antibodies were raised in rabbits by injecting biotin-bovine serum albumin conjugate. Neither the protomer nor the polymer of rat mammary-gland acetyl-CoA carboxylase formed precipitin bands with the anti-biotin. By virtue of its ability to bind biotin (apparent binding constant for free biotin about $1\mu\text{M}$), the anti-biotin inhibited the carboxylase activity under certain conditions. This property of the antibody was employed to detect the ligand-induced changes affecting the biotinyl group in different conformational states of mammalian carboxylase. Depending on the ligand present, the biotinyl group in the protomeric form was either accessible or inaccessible to the antibody. The biotinyl group of the protomer generated by a relatively high concentration of NaCl (0.5 M) reacted with the antibody, and the antibody-carboxylase complex could not be converted into active enzyme by citrate. Further experiments showed that citrate failed to induce polymerization in this protomer-antibody complex and that anti-biotin could be displaced rapidly from this complex with excess of biotin. The resulting protomer was converted into the polymeric state on citrate addition, with parallel regain of enzyme activity. In the presence of $\text{ADP} + \text{Mg}^{2+}$, $\text{ATP} + \text{Mg}^{2+}$ or $\text{ATP} + \text{Mg}^{2+} + \text{HCO}_3^-$, however, the enzyme remained as a protomer, but its configuration was such that the biotinyl group was essentially inaccessible to the antibody. Likewise, the biotinyl group of the different polymeric forms of the carboxylase ($s \sim 30\text{--}45\text{S}$) engendered by phosphate, malonyl-CoA, acetyl-CoA or citrate remained essentially inaccessible, since their activity was minimally affected by the anti-biotin. In the presence of 0.15 M-NaCl, the phosphate-induced polymer reverted to a $\sim 19\text{S}$ form with concomitant appearance of anti-biotin-sensitivity, whereas the other polymeric forms remained unaffected under similar experimental conditions.

Acetyl-CoA carboxylase (EC 6.4.1.2), a biotinyl enzyme, catalyses the formation of malonyl-CoA as shown:

Besides these tricarboxylic acids, certain anions (e.g. phosphate, sulphate), acetyl-CoA and high protein concentration also favour polymerization of the

The enzyme of animal origin exists mainly as an inactive protomer, which in the presence of citrate or isocitrate is converted readily into the enzymically active polymer (for review see Lane *et al.*, 1974).

carboxylase protomer into an intermediate size ($\sim 30\text{S}$) or into the large polymeric state (45 S). In contrast, high pH, low protein concentration, treatment with NaCl (0.5 M) or carboxylation of the

enzyme molecules to enzyme-biotin- CO_2^- leads to disaggregation of the chicken liver carboxylase polymers (Lane *et al.*, 1974; Gregolin *et al.*, 1968b).

As established for other biotin-containing enzymes (Lynen *et al.*, 1961; Lane *et al.*, 1974), the overall reaction (iii) catalysed by acetyl-CoA carboxylase proceeds via two half-reactions (i) and (ii). Previous studies with the chicken liver enzyme showed that citrate enhanced the rates of catalysis of both the half-reactions of acetyl-CoA carboxylation (Gregolin *et al.*, 1968a). Since biotin participates in the biotin carboxylase (i) and the carboxytransferase (ii) reactions, the citrate-induced conformational change was proposed to occur in the vicinity of this group with attendant alterations in the catalytic efficiency of the enzyme molecules.

The highly purified rat mammary-gland carboxylase also exists in discrete protomeric and polymeric forms (F. Ahmad *et al.*, 1978). Either of the forms can be obtained by treating the enzyme with a number of structurally unrelated compounds. This phenomenon prompted us to inquire: (a) does the protomer-to-polymer transition affect the biotinyl group, and, if so, (b) do those compounds that elicit a similar type of hydrodynamic change in the carboxylase molecules perturb the biotinyl group identically? To answer these questions we employed biotin-binding antibodies, which have previously been shown (Berger, 1975) to inhibit the activity of biotin-containing enzymes, methylmalonyl-CoA:pyruvate carboxyltransferase (EC 2.1.3.1) and avian liver pyruvate carboxylase (EC 6.4.1.1). Our results show that the ligand-induced alterations affecting the biotinyl group of the mammalian carboxylase can be readily detected by using these antibodies. Moreover, these antibodies were able to detect not only differences between protomer and polymer but also subtle differences between various protomers/polymers generated by different ligands.

A preliminary report of these studies has been presented (P. M. Ahmad *et al.*, 1978).

Materials and methods

Materials

ATP, CoA and NADPH were purchased from P-L Biochemicals (Milwaukee, WI, U.S.A.). Sigma Chemical Co. (St. Louis, MO, U.S.A.) was the source for sucrose, bovine serum albumin (essentially fatty acid-free) and dithiothreitol. Freund's complete adjuvant was purchased from Difco Laboratories (Detroit, MI, U.S.A.), $\text{Ba}^{14}\text{CO}_3$ (58 mCi/mmol) from ICN (Irvine, CA, U.S.A.), D-[carbonyl- ^{14}C]biotin (45 mCi/mmol) from Amer-sham/Searle Corp. (Arlington Heights, IL, U.S.A.) and Omnifluor from New England Nuclear Corp. (Boston, MA, U.S.A.). All other chemicals were of the highest purity commercially available. Acetyl-

CoA was prepared by the method of Simon & Shemin (1953). Its concentration was determined enzymically (Tubbs & Garland, 1969).

Covalent attachment of biotin to bovine serum albumin

Biotin was covalently linked to bovine serum albumin essentially as described by Berger (1975). These preparations contained approx. 12–15 μmol of biotin/ μmol of serum albumin. The concentration of biotin was determined by the radioisotopic method.

Preparation of biotin-binding antibodies

An emulsion consisting of 0.5 ml of Freund's complete adjuvant and 0.5 ml of bovine serum albumin-biotin complex (2 mg/ml) was injected into the foot-pads of two male New Zealand White rabbits. At 2-week intervals further serum albumin-biotin complex (1 mg/ml in sterile 0.9% NaCl) was injected intravenously. Then, 8 days after the fourth injection, blood samples were collected, kept for 1 h at 37°C and then stored at 4°C overnight. The serum was separated by centrifugation at 2000g, and the immunoglobulin was precipitated by the addition of a saturated solution of $(\text{NH}_4)_2\text{SO}_4$ to give 40% (w/v) saturation. The precipitated protein was collected by centrifugation at 27000g, dissolved in 50 mM-Tris/HCl buffer, pH 7.5, containing 0.15 M-NaCl and dialysed against the same buffer for 16–18 h at 4°C. The dialysed antiserum was divided into small portions and stored at –20°C.

Ouchterlony double-diffusion analysis

Double-diffusion analyses were performed by the method of Ouchterlony & Nilsson (1978), with minor modifications (F. Ahmad *et al.*, 1978).

Purification of rat mammary-gland acetyl-CoA carboxylase

Acetyl-CoA carboxylase from rat lactating mammary gland was purified by the method of F. Ahmad *et al.* (1978). The purified enzyme had a specific activity of approx. 15 units/mg of protein. Highly purified preparations of the carboxylase were stored in buffer containing 50 mM-imidazole/HCl, pH 6.5, 7 mM- β -mercaptoethanol, 0.1 mM-EDTA, 20 mM-potassium citrate and 20% (v/v) glycerol at –70°C for over a 2-year period with no noticeable loss in activity.

Buffers employed

The following buffers were used frequently: buffer A (50 mM-imidazole/HCl, pH 7.5, containing 0.1 mM-EDTA and 20% glycerol); buffer B (buffer A containing 1 mg of bovine serum albumin/ml). Deviations from their compositions are described in the text.

Preparation of different interconvertible forms of acetyl-CoA carboxylase

A sample of the enzyme was dialysed or diluted 10-fold in buffer A and kept at 27°C for up to 72 h. Further dilution was made in buffer A containing either 25 mM-sodium citrate for the polymer or 0.5 M-NaCl for the protomer. For the latter, the concentration of NaCl was decreased to 0.15 M or less after 30 min at 27°C and the enzyme preparations were used immediately for inhibition studies. Deviations from this procedure are detailed in the legends to the Tables or Figures.

Determination of acetyl-CoA carboxylase activity

Different forms of the enzyme were preincubated, in quadruplicate, with the desired ligand dissolved in buffer B $\pm$ NaCl for 10 min at 27°C. Anti-biotin serum was then added to samples 1 and 2, and buffer was added to the control tubes 3 and 4. After an additional 10 min incubation at 27°C, portions of antibody-treated or of control enzyme were added to the assay tubes so that the final concentrations of the ingredients equalled: 50 mM-imidazole/HCl buffer, pH 7.5, 30 mM-citrate, 24 mM-MgCl₂ and 0.05 mg of bovine serum albumin. This was followed by the immediate addition of 0.75 mM-acetyl-CoA, 25 mM-NaH¹⁴CO₃ (400–600 c.p.m./nmol) and 7.2 mM-ATP. In each case the total volume was 0.1 ml. The contents were incubated for 5 min at 37°C and the reaction was halted by the addition of 0.025 ml of 4 M-HCl. A 0.1 ml portion of the assay contents was transferred to a scintillation vial, dried and the radioactivity measured as described previously (F. Ahmad *et al.*, 1978). The variation between duplicate samples was within $\pm 5\%$. Various determinations were performed at least three times with two to four different enzyme preparations and the immune globulin fraction derived from two different rabbits.

In most of the experiments described, the anti-biotin concentration was selected so that the activity remaining in the protomer (after citrate addition) was approx. 40–50% of the control samples.

Assay conditions were selected to achieve a reaction rate proportional to the amount of enzyme added and the duration of incubation. One unit of enzyme activity equals the amount of carboxylase protein catalysing the formation of 1 μ mol of malonyl-CoA/min at 37°C.

Density-gradient centrifugation

Preparation of sucrose and glycerol density gradients followed the method of Martin & Ames (1961). Each gradient had a total volume of 4.6 ml, and sedimentation coefficients were determined after centrifuging at 40 000 rev./min for 2.5 h at 23°C in an SW 50L rotor of a Beckman L2-65B ultracentrifuge. Fractions (15 drops each) were collected

manually and samples were assayed for acetyl-CoA carboxylase activity as described above.

Protein determination

Protein was determined by the method of Lowry *et al.* (1951), with bovine serum albumin as standard.

Results

Reaction of anti-(bovine serum albumin–biotin complex) with bovine serum albumin–biotin complex and bovine serum albumin

Bovine serum albumin containing covalently linked biotin, when injected into rabbits, elicited antibodies that reacted with the modified serum albumin. The immune γ -globulin fraction was found to be highly specific in its interaction with bovine serum albumin–biotin complex, since during immunodiffusion studies a precipitin band was observed at 1 μ g of complex/well but no band formed when bovine serum albumin alone was used at concentrations up to 100 μ g/well (Fig. 1). Faint precipitin bands appeared at serum albumin concentrations of 150–200 μ g/well.

Fig. 1. Ouchterlony double-diffusion analysis of biotin-binding antibodies with bovine serum albumin and biotin–bovine serum albumin conjugate

Double-diffusion analyses were conducted as described in the text. The centre well contained 106 μ g of anti-(bovine serum albumin–biotin complex). Outer wells contained the following: 1 and 4, 1 μ g of bovine serum albumin–biotin conjugate; 2 and 5, 10 μ g of bovine serum albumin; 3 and 6, 100 μ g of bovine serum albumin.

When biotin was added to the agarose before immunodiffusion, it resulted in deletion of the antigen-antibody precipitate. However, when bovine serum albumin was incorporated into the agarose the precipitin band still formed. These results indicated that antibodies specific to the biotinyl group had been formed. Although these antibodies did not form precipitin bands with the protomeric and polymeric forms of acetyl-CoA carboxylase, they rapidly inactivated highly purified rat mammary-gland acetyl-CoA carboxylase under certain conditions.

Interaction of the protomeric and polymeric forms of acetyl-CoA carboxylase with biotin-binding antibodies

The protomeric and polymeric forms of acetyl-CoA carboxylase were incubated separately with anti-biotin under conditions maintaining the enzyme

Fig. 2. Effects of biotin-binding antibodies on the protomeric and polymeric forms of mammalian acetyl-CoA carboxylase

Acetyl-CoA carboxylase (specific activity ~14 units/mg) was diluted 10-fold in buffer A containing 1 mM-EDTA. Further dilution was made in the same buffer plus 25 mM-citrate (for polymer) or 0.5 M-NaCl (for protomer). After incubation at 27°C for 30 min, enzyme samples (0.2 munit) were incubated further for 10 min at 27°C in a mixture (0.1 ml) containing buffer B and various amounts of anti-biotin or non-immune rabbit serum. During the incubations citrate was maintained at 25 mM for the polymer, whereas NaCl concentration was lowered to 0.15 M for the protomer. The enzymic activity was determined on samples of the antibody treated or control samples as given in the Materials and methods section. All assays were performed in duplicate, with agreement of $\pm 5\%$. Controls with buffer instead of antiserum were run in duplicate for each variable. ▲, Protomer + non-immune serum; ○, polymer + non-immune serum; ●, protomer + anti-biotin; ■, polymer + anti-biotin.

in either the protomeric or the polymeric state. After incubation of the enzyme-antibody mixtures, citrate was added and the enzymic activity was determined. The anti-biotin (Figs. 2 and 3), when incubated with the protomeric form, caused inhibition of carboxylase activity. The loss of enzymic activity occurs because the antibody binds the biotinyl group, thereby preventing its participation in catalysis. In contrast with the protomer, the polymeric form produced by citrate was inhibited to a much smaller extent by the antibody. For example, 3 μg of the immune serum led to 70% inhibition in enzyme activity with the protomer and only 15% when the polymer was employed (Fig. 2). The rate of anti-biotin-dependent inhibition followed first-order kinetics (Fig. 3), and the loss in enzyme activity was exponentially related to antibody concentration (Fig. 2). Independent experiments showed that the non-immune serum had no deleterious effect on the carboxylase activity.

Effect of low-molecular-weight compounds on the interaction of anti-biotin with acetyl-CoA carboxylase

The accessibility of the biotinyl group of acetyl-CoA carboxylase to anti-biotin was studied in the presence of different low-molecular-weight ligands. The presence of 20 mM- or 50 mM-phosphate

Fig. 3. Time course of inactivation of acetyl-CoA carboxylase by biotin-binding antibodies

The protomeric (●) and polymeric forms (■) of the enzyme (0.2 munit) described in the legend to Fig. 2 were incubated with 3 μg of biotin-binding antibodies for various lengths of time at 27°C. The enzyme activity was determined as given in the Materials and methods section.

Table 1. *Effects of NaCl on the inhibition of mammalian acetyl-CoA carboxylase activity by anti-biotin in the presence of citrate or phosphate*

Acetyl-CoA carboxylase (1.3 mg/ml) was dissolved in and dialysed for 1 h at 4°C against buffer A containing 1 mM-EDTA. Two samples of the dialysed enzyme were separately diluted 10-fold (a) in the above buffer and (b) in the above buffer containing 0.5 M-NaCl. Both the samples were incubated for 30 min at 27°C. Equal volumes (10 μ l) of the diluted enzyme in buffers with or without NaCl were transferred to quadruplicate sets of tubes containing buffer B. In the series treated with NaCl, its final concentration was 0.15 M. After addition of citrate or phosphate, the contents of the tubes were incubated at 27°C for 10 min. The tubes were incubated at 27°C for a further 10 min period after duplicate sets had received either anti-biotin (10 μ g) or buffer. Controls were prepared from enzyme solution that had not been incubated with 0.5 M-NaCl. Acetyl-CoA carboxylase activity was determined as described in Materials and methods section. Sedimentation coefficients relative to catalase and thyroglobulin were determined, by centrifuging samples, treated as detailed above, over 10–30% (v/v) glycerol gradients prepared in buffers containing ligands at concentrations shown. Density-gradient centrifugation was done as described in the Materials and methods section.

Expt. no.	Addition	Inhibition (%)		Sedimentation coefficient (S)	
		–NaCl	+NaCl	–NaCl	+NaCl
1	Control	46	65	—	13
2	Citrate (20 mM)	20	22	45	31
3	Phosphate (20 mM)	21	53	32	19
4	Phosphate (50 mM)	3	40	—	—

diminished the interaction between the biotinyl group of the protomer with the anti-biotin (Table 1). This phenomenon is similar to that observed with citrate (Figs. 2 and 3). The citrate-induced change was not reversed in the presence of 0.15 M-NaCl (Expt. 2 in Table 1); however, the protective effect afforded by phosphate was nearly abolished (compare Expts. 3 and 4 with Expt. 1 in Table 1) when 0.15 M-NaCl was included during incubation of the enzyme with anti-biotin.

The relationship between sedimentation behaviour and anti-biotin-dependent inhibition of carboxylase activity in the presence of various ligands was also investigated. The carboxylase protomer (13–14 S) was converted into the polymeric state in the presence of phosphate (~32 S) and citrate ($\geq$ 45 S). Addition of 0.15 M-NaCl to the phosphate-induced polymer decreased the sedimentation coefficient to ~19 S. Addition of an identical salt concentration decreased the sedimentation coefficient of the citrate-induced polymer to ~31 S, suggesting that some disaggregation occurs in this case also. However, enzyme activity was inhibited only when the slower-sedimenting species ($\leq$ 19 S) was incubated with the antibody. Thus the phosphate-induced perturbation affecting the biotinyl group could be distinguished from the citrate-mediated change, which was more stable under these experimental conditions.

The results of kinetic studies have shown that CoA is competitive with acetyl-CoA, but binds somewhat poorly to rat mammary-gland carboxylase (K_i 2–3 mM) (J. Rodriguez & F. Ahmad,

unpublished work). In the presence of CoA (0.25–1 mM) the protomer was inhibited by the antibody almost to the same extent as in the absence of CoA (Table 2). The sedimentation coefficient of the protomer increased to ~18 S in the presence of CoA, which is consistent with the formation of a dimer. Since this sedimenting form of the carboxylase is inhibited by the antibody, it may be concluded that the conformation of the biotinyl group in the putative dimer is not constrained sufficiently to exclude binding of anti-biotin to biotin. Similarly in phosphate the anti-biotin inhibited the enzyme that had been disaggregated by NaCl (~19 S; Expt. 3 in Table 1).

In the presence of as high a concentration as 100 μ M-malonyl-CoA the rat mammary-gland acetyl-CoA carboxylase behaved as an intermediate-size polymer (28–30 S), and the enzyme sustained minimal loss in activity during incubation with the antibody in the presence or in the absence of NaCl (Table 2). The biotinyl group of the carboxylase was carboxylated after incubation with either Mg^{2+} -ATP + HCO_3^- or with malonyl-CoA (results not presented). The enzyme remained as a protomer in the presence of Mg^{2+} -ATP + HCO_3^- , but it was converted into an intermediate-size polymer in the presence of malonyl-CoA. This observation represents a distinct difference between rat mammary-gland carboxylase and the chick liver enzyme, since addition of as little as 10 μ M-malonyl-CoA to the polymeric form of chick liver carboxylase causes instantaneous disaggregation (Lane *et al.*, 1974).

Table 2. *Effects of CoA, acetyl-CoA and malonyl-CoA on the inhibition of acetyl-CoA carboxylase activity by anti-biotin*

A sample of acetyl-CoA carboxylase (13.2 mg/ml) was diluted 7-fold in buffer A containing 1 mM-EDTA. A portion of the carboxylase solution was diluted 20-fold further in similar buffer containing 0.5 M-NaCl and then incubated for 30 min at 27°C. Samples of this protomer (0.47 µg of protein) were preincubated at 27°C for 10 min in a final volume of 50 µl of buffer B containing 0.1 M-NaCl and CoA, acetyl-CoA or malonyl-CoA at concentrations shown. Anti-biotin (4.5 µg) or buffer was added to duplicate tubes of each series, and, after additional incubation at 27°C for 10 min, samples were removed and assayed for carboxylase activity. Sedimentation coefficients were determined as given in the legend to Table 1. When experiments were conducted in the presence of malonyl-CoA, the volume of the preincubation mixture was 0.1 ml; the amount of antibody added equalled 10 µg and the carboxylase protomer was 13 µg. Inhibition obtained in control tubes (with malonyl-CoA omitted) was 58.4%.

Expt. no.	Addition	Concentration	Inhibition (%)	Sedimentation coefficient (S)
1	Control	—	62	13
2	CoA	0.25 mM	56	—
		0.5 mM	56	—
		1.0 mM	57	18
3	Acetyl-CoA	0.25 mM	33	—
		0.5 mM	38	44
		0.75 mM	27	—
		1.00 mM	21	—
4	Malonyl-CoA	5 µM	18	—
		10 µM	13	30
		25 µM	24	—
		100 µM	20	28

Table 3. *Effects of Mg^{2+} , $Mg^{2+} + AMP$, $Mg^{2+} + ADP$, $Mg^{2+} + ATP$ and $Mg^{2+} + ATP + HCO_3^-$ on anti-biotin-dependent inhibition of acetyl-CoA carboxylase activity*

Acetyl-CoA carboxylase (1.3 mg/ml) was dialysed for 1 h at 4°C against buffer A containing 1 mM-EDTA. A portion was diluted 10-fold in the same buffer containing 0.5 M-NaCl and incubated at 27°C for 30 min. Quadruplicate portions (10 µl) of the protomer thus formed were preincubated in a final volume of 100 µl containing buffer B and ligands at concentrations shown with either low (0.045 M) or high concentration (0.145 M) of NaCl. After incubation at 27°C for 10 min either buffer or anti-biotin (10 µg) was added to duplicate tubes and the contents were incubated for an additional 10 min period at 27°C. The carboxylase activity was measured on a portion of these samples as detailed in the Materials and methods section. The sedimentation coefficients were determined on enzyme samples preincubated with different ligands in the presence of low salt concentration.

Expt. no.	Additions	Inhibition (%)		Sedimentation coefficient (S)
		0.045 M-NaCl	0.145 M-NaCl	
1	Control	50	65	13
2	MgCl ₂ (7.5 mM)	33	55	14
3	MgCl ₂ (7.5 mM) + AMP (7.5 mM)	59	78	14
4	MgCl ₂ (7.5 mM) + ADP (7.5 mM)	13	33	14
5	MgCl ₂ (7.5 mM) + ATP (7.5 mM)	18	33	13
6	MgCl ₂ (7.5 mM) + ATP (7.5 mM) + HCO ₃ ⁻ (7.5 mM)	16	19	13
7	HCO ₃ ⁻ (7.5 mM)	58	67	—
	HCO ₃ ⁻ (15 mM)	38	61	14
	HCO ₃ ⁻ (30 mM)	34	55	—

The enzyme depolymerized by high concentration of NaCl (0.5 M) remains as a protomer ($s \sim 13$ – $16S$) even though the salt concentration is lowered to either 0.15 M or 0.045 M. It also maintained this sedimentation characteristic when its solution in 0.45 M-NaCl was supplemented with Mg^{2+} , $Mg^{2+} + AMP$, $Mg^{2+} + ADP$, $Mg^{2+} + ATP$, $Mg^{2+} + ATP + HCO_3^-$ or HCO_3^- (Table 3). But,

unlike the situation with the protomer in 0.150 M-NaCl (Figs. 2 and 3), the protomer in the presence of several of these compounds became considerably less susceptible to antibody-dependent inhibition (Table 3). This was particularly evident when the enzyme was treated with the antibody in the presence of $Mg^{2+} + ADP$, $Mg^{2+} + ATP$ or $Mg^{2+} + ATP + HCO_3^-$. It is noteworthy that $Mg^{2+} + AMP$

did not afford protection. Therefore it appears that only those nucleotides that serve as substrate (Mg^{2+} -ATP) or product (Mg^{2+} -ADP) of the carboxylase reaction constrain its conformation such that its biotinyl group is prevented from interacting with the anti-biotin.

Biotin-binding antibodies prevent the protomer from undergoing citrate-induced polymerization

On addition of excess of biotin, the anti-biotin-inhibited protomer was rapidly reactivated to about 60–70% of the original activity (see below). This property of the antibody–enzyme complex was exploited to determine citrate's effect on the anti-biotin-treated protomer. In these experiments, the carboxylase protomer was incubated with sufficient antibody to produce 70–80% inhibition of enzyme activity. Two samples of the antibody-inhibited enzyme were separately subjected to density-gradient centrifugation in the presence and in the absence of citrate (Fig. 4). The different sedimenting

forms of the carboxylase were detected by measuring enzyme activity on fractions to which biotin had been added. In both cases, most of the enzyme applied to the gradient sedimented with a sedimentation coefficient of 13–16S, indicating that the antibody-treated protomer could not be converted into the polymeric state by citrate.

Results given in Fig. 4 also show that addition of biotin first, then citrate, to the inactivated enzyme produced the polymeric form of the carboxylase ($s \geq 32$ S). Therefore displacement of the antibody by biotin from the enzyme–antibody complex yielded carboxylase that appeared similar to the untreated protomer in its ability to undergo polymerization and activation by citrate.

Re-activation of anti-biotin-inhibited acetyl-CoA carboxylase and determination of the binding constant of biotin for biotin-binding antibodies

To determine if the antibody-inhibited enzyme can be re-activated by adding excess of biotin, the

Fig. 4. Sedimentation behaviour of the antibody-treated acetyl-CoA carboxylase in the presence and in the absence of citrate

The carboxylase protomer (185 μg) was incubated for 10 min at 27°C in a mixture (total volume 0.1 ml) containing 50 mM-imidazole/HCl buffer, pH 7.5, 1.0 mM-EDTA, 0.5 mM-dithiothreitol, 0.15 M-NaCl and 1.5 mg of anti-biotin. The inhibited enzyme (~80% loss in enzyme activity) was divided into three 30 μl portions, which were treated as follows. (1) Control (●): volume adjusted to 0.2 ml with the buffer described above. (2) Addition of citrate (■): sufficient citrate, dissolved in 50 mM-imidazole/HCl buffer, pH 7.5, was added to obtain a final concentration of 30 mM in a total volume of 0.2 ml. (3) Addition of biotin (Δ): biotin (to 1 mM concentration) was added and the sample was incubated at 27°C for 5 min, and then citrate was added to 30 mM concentration in a final volume of 0.2 ml. The samples were layered over separate 5–20% (w/v) sucrose gradients prepared by dissolving sucrose in 50 mM-imidazole/HCl buffer, pH 7.5, containing 1 mM-EDTA, 0.5 mM-dithiothreitol and either 0.15 M-NaCl (sample 1) or 0.15 M-NaCl + 30 mM-citrate (samples 2 and 3). The tubes were centrifuged at 40 000 rev./min for 2 h at 25°C in an L2 65B ultracentrifuge. After fractionation, 0.7 μg of biotin was added to 20 μl portions of each fraction. After citrate addition and incubation at 27°C for 10 min, the enzyme activity was determined as given in the Materials and methods section.

Fig. 5. Reversal of inhibition of antibody-treated acetyl-CoA carboxylase by biotin

The protomeric form of acetyl-CoA carboxylase (10 munits) was incubated at 27°C for 15 min with 20 μ g of immune γ -globulin. Simultaneously with biotin addition (2 μ g) to the major portion, a separate portion of the enzyme-antibody mixture was removed, incubated immediately with citrate and then assayed for enzyme activity. This value established the degree of inhibition achieved, corresponding to 80% loss of activity. Samples from the antibody-treated enzyme to which biotin had been added were removed at indicated times and assayed for enzyme activity after citrate addition.

following experiment was performed. The protomer, in the presence of 0.15 M-NaCl, was treated with an amount of the antibody producing approx. 80% inhibition in enzyme activity. The rate of regain in enzymic activity was then determined after addition of biotin. As shown in Fig. 5, enzymic activity increased almost immediately after biotin addition and did not increase further on more prolonged incubation. Despite a large excess of biotin, and increased time of incubation, the degree of re-activation of antibody-inhibited enzyme did not exceed 60–70% of the original activity. These results indicated that either a small amount of acetyl-CoA carboxylase underwent denaturation and thus some activity was irreversibly lost, or that there existed another population of antibodies possessing much higher affinity for biotin. Such antibodies might bind the biotinyl group of the carboxylase so tightly that reversal of inhibition might not be achieved by the addition of excess of biotin.

Inhibition of enzyme activity was almost linear over an extremely narrow concentration range of the antibody (up to about 20–25% inhibition). Therefore within these limits enzyme inhibition could be

Fig. 6. Determination of the binding constant of biotin for biotin-binding antibodies

Samples of anti-biotin serum containing 0.48 μ g of protein were preincubated with various amounts of biotin for 30 min at 37°C in a mixture (30 μ l) containing buffer A plus 1.0 mM-EDTA. A constant amount of the carboxylase protomer (0.8 munit) and bovine serum albumin (final concentration 2 mg/ml) were added to each tube containing the biotin/anti-biotin mixture and further incubated at 37°C for 30 min. After citrate addition, the enzymic activity was determined as given in the Materials and methods section. Data were plotted according to the procedure of Dixon & Webb (1964). The amount of antiserum used was selected to avoid antibody excess. All determinations were done in duplicate. The value of the control incubation is indicated by the arrow.

used as a measure of the concentration of free antibody present in antibody/biotin mixtures, allowing measurement of the binding constant of antibody for biotin.

The results shown in Fig. 6 represent an example of the binding studies conducted with these antibodies. The amounts of antibody and acetyl-CoA carboxylase were selected so that inhibition of enzyme activity did not exceed 20–25%. Various amounts of biotin were incubated with the antibody, the acetyl-CoA carboxylase protomer was added to this mixture and the enzyme activity was determined after additional incubation. The enzyme activity increased with increasing biotin, indicating that the antibody was being neutralized by the biotin. The data were replotted on a double-reciprocal plot, as shown in the inset of Fig. 6. An apparent binding constant of 1 μ M for biotin to antibody was calculated (Dixon & Webb, 1964).

Discussion

The results presented above clearly show that biotin-binding antibodies can detect the ligand-induced alterations affecting the biotinyl group in both the protomeric and polymeric forms of mammalian acetyl-CoA carboxylase. The interaction of anti-biotin with acetyl-CoA carboxylase must be highly specific, probably involving its biotinyl group only, since the bovine serum albumin that carried the biotinyl group during antibody production does not possess antigenic determinants in common with the carboxylase. Furthermore, the ease with which enzymic activity could be determined, the rapidity of the reaction of antibody with the enzyme and the extremely small amounts of enzyme and antibody required made enzyme inactivation a very useful tool for detection of conditions perturbing the biotinyl group of the carboxylase.

The anti-biotin failed to produce precipitin bands when tested against the protomeric and polymeric forms of mammalian acetyl-CoA carboxylase containing biotin (50 μ g of acetyl-CoA carboxylase/well, corresponding to approx. 50 ng of biotin) equivalent to or exceeding that in the bovine serum albumin-biotin conjugate that gave positive precipitin tests. With the carboxylase polymer the lack of precipitin band formation indicates that the enzyme's biotinyl group is not accessible to the anti-biotin. Our findings that the polymeric form of the carboxylase is inhibited poorly by the anti-biotin strengthens this suggestion. However, a different explanation is offered for the lack of precipitin band formation when the protomer engendered by high salt concentration reacts with the anti-biotin. The protomeric form of rat mammary-gland acetyl-CoA carboxylase is believed to consist of a single polypeptide chain of M_r 240 000–260 000 containing one molecule of biotin (F. Ahmad *et al.*, 1978). The anti-biotin antibodies involved in these reactions are probably bivalent immunoglobulin G molecules. Therefore each of the immunoglobulin G molecules could bind at most two molecules of the carboxylase protomer. Clearly, such an arrangement of the antigen-antibody molecules would preclude lattice formation, a prerequisite for immunoprecipitation. This interpretation is supported by the results obtained by Hohman & Stadtman (1978), who found that the mono-adenylated glutamine synthetase (EC 6.3.1.2) of *Escherichia coli* was not precipitated by anti-AMP antibodies whereas these antibodies quantitatively precipitated the extensively modified enzyme (11 AMP groups per dodecamer).

Citrate accelerates the rate of catalysis of both the partial reactions (i) and (ii) of acetyl-CoA carboxylase (Lane *et al.*, 1974). As the mammalian carboxylase acquires polymeric and active con-

formation, its activity is least inhibited by biotin-binding antibodies. In a previous report, avidin, the biotin-binding protein, also did not inhibit the activity of the polymeric form of avian liver carboxylase (Lane *et al.*, 1974). Therefore it appears that the addition of citrate causes the biotinyl group of the carboxylase to orient itself in such a way that it is protected from interacting with biotin-binding proteins.

A number of ligands ($Mg^{2+} + ADP$, $Mg^{2+} + ATP$, $Mg^{2+} + ATP + HCO_3^-$) when added to the protomer allowed it to remain as a protomer or caused it to acquire either the intermediate (phosphate, malonyl-CoA) or the large polymeric state (acetyl-CoA, citrate). Regardless of the hydrodynamic species engendered by these ligands, the biotin of the enzyme in the presence of these ligands was mostly protected from interacting with the anti-biotin. Since these compounds serve as substrates, products or activators of acetyl-CoA carboxylase, their binding probably signals the biotinyl group to undergo translocation, perhaps to an active-site crevice consisting of the biotin carboxylase (i) and the carboxytransferase (ii) subsites. The transfer of the carboxy group between these two subsites will be facilitated by positioning of the biotinyl group at such a locus. Furthermore, in such an active-site crevice the biotinyl group of the enzyme might be shielded, perhaps by neighbouring groups, and hence inaccessible to the antibody.

Besides the protomer that does not react with the antibody, the mammalian carboxylase could be converted into a protomeric state that reacted rapidly with the antibody. Thus in this conformational state the biotinyl group of the carboxylase appears to be located in an exposed position, perhaps at the enzyme surface. This phenomenon is analogous to that described for methylmalonyl-CoA:pyruvate carboxytransferase. Electron-microscopic analysis of methylmalonyl-CoA:pyruvate carboxytransferase-avidin complexes revealed in a convincing manner that the biotinyl group of this enzyme was located on an exposed and extended polypeptide chain (Green *et al.*, 1972).

The antibody-treated protomer failed to be polymerized by citrate. It is not yet clear whether binding of citrate is prevented as a result of steric hindrance or whether the citrate can still bind to the enzyme-anti-biotin complex but can no longer induce the productive conformational change that normally leads to polymerization and activation of acetyl-CoA carboxylase protomer.

The anti-biotin antibodies may also prove valuable in exploring the ligand-induced perturbations affecting the biotinyl groups of other biotin-containing enzymes whose activities are allosterically regulated, e.g. pyruvate carboxylase (Utter *et al.*, 1975). In addition, the availability of these anti-

bodies may provide a practical method for purifying biotin-containing enzymes by affinity chromatography. This is particularly indicated, since the antibody used in these investigations could be displaced rapidly from the enzyme-antibody complex by free biotin.

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