Function of *Escherichia coli* Biotin Carboxylase Requires Catalytic Activity of Both Subunits of the Homodimer*

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Biotin carboxylase catalyzes the ATP-dependent carboxylation of biotin and is one component of the multienzyme complex acetyl-CoA carboxylase that catalyzes the first committed step in fatty acid synthesis. The *Escherichia coli* biotin carboxylase is readily isolated from the other components of the acetyl-CoA carboxylase complex such that enzymatic activity is retained. The three-dimensional structure of biotin carboxylase, determined by x-ray crystallography, demonstrated that the enzyme is a homodimer consisting of two active sites in which each subunit contains a complete active site. To understand how each subunit contributes to the overall function of biotin carboxylase, we made hybrid molecules in which one subunit had a wild-type active site, and the other subunit contained an active site mutation known to significantly affect the activity of the enzyme. One of the two genes encoded a poly-histidine tag at its N terminus, whereas the other gene had an N-terminal FLAG epitope tag. The two genes were assembled into a mini-operand that was induced to give high level expression of both enzymes. “Hybrid” dimers composed of one subunit with a wild-type active site and a second subunit having a mutant active site were obtained by sequential chromatographic steps on columns of immobilized nickel chelate and anti-FLAG affinity matrices. *In vitro* kinetic studies of biotin carboxylase dimers in which both subunits were wild type revealed that the presence of the N-terminal tags did not alter the activity of the enzyme. However, kinetic assays of hybrid dimer biotin carboxylase molecules in which one subunit had an active site mutation (R292A, N290A, R238Q, or E228R) and the other subunit had a wild-type active site resulted in 39-, 28-, 94-, and 285-fold decreases in the activity of these enzymes, respectively. The dominant negative effects of these mutant subunits were also detected *in vivo* by monitoring the rate of fatty acid biosynthesis by $^{14}$C-labeled labeling of cellular lipids. Expression of the mutant biotin carboxylase genes from an inducible arabinose promoter resulted in a significantly reduced rate of fatty acid synthesis relative to the same strain that expressed the wild-type gene. Thus, both the *in vitro* and *in vivo* data indicate that both subunits of biotin carboxylase are required for activity and that the two subunits must be in communication during enzyme function.

* This work was supported in part by National Institutes of Health Grants AI15650 (to J. E. C.) and GM51261 (to G. L. W.). The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked “advertisement” in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

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Escherichia coli* acetyl-CoA carboxylase catalyzes the first committed and rate-controlling step in fatty acid biosynthesis (1). The overall reaction catalyzed by acetyl-CoA carboxylase proceeds via two half-reactions as shown in the scheme in Fig. 1. To carry out this two-step reaction acetyl-CoA carboxylase requires three distinct components: biotin carboxylase, biotin carboxyl carrier protein, and carboxyltransferase. The biotin carboxylase component catalyzes the first half-reaction in Fig. 1, which is an ATP-dependent carboxylation of the vitamin, biotin. *In vivo*, biotin is covalently attached to the biotin carboxyl carrier protein designated as BCCP in Fig. 1. The second half-reaction, the transfer of the carboxyl group from carboxybiotin to acetyl-CoA to make malonyl-CoA, is catalyzed by the carboxyltransferase component. In mammals, all three acetyl-CoA carboxylase components reside on one polypeptide chain (2). However, in the prokaryotic (and chloroplast) forms of the enzyme, the components are a series of individual proteins that form a complex. The component proteins can be isolated from one another with the biotin carboxylase and carboxyltransferase components retaining enzymatic activity in their respective half-reactions (3). Moreover, both the biotin carboxylase and carboxyltransferase components will utilize free biotin as substrate *in vitro* (3). These features have made the *E. coli* acetyl-CoA carboxylase the prototype for the study of other biotin-dependent carboxylases.

The *E. coli* biotin carboxylase component encoded by the *accC* gene has become the model system for studying biotin-dependent carboxylation reactions for several reasons. First, the gene encoding the enzyme has been cloned, sequenced, and overexpressed (4, 5). Second, the crystal structure of the enzyme has been determined and remains the only three-dimensional model of a biotin-dependent carboxylase (6, 7). The structural studies of biotin carboxylase confirmed the enzyme is a homodimer having two well separated active sites that do not contain residues from both subunits (also referred to as a shared active site) as seen with other multisubunit enzymes such as aspartate transcarbamylase (8) and ribulose-1,5-bisphosphate carboxylase (9). Instead, each subunit of biotin carboxylase contains a complete active site. This raises the question: why does biotin carboxylase function as a homodimer? Kinetic analyses have shown no signs of cooperative behavior for any of the three substrates (ATP, bicarbonate, or biotin), suggesting that there is no communication between the two subunits, yet the enzyme exists as a homodimer. To better understand the subunit structure of biotin carboxylase, the studies in this report address the following questions: (i) Are both subunits of the biotin carboxylase homodimer required for activity or is one subunit sufficient for catalysis? (ii) Similarly, is there communication between the subunits of biotin carboxylase or do they act independently? (iii) Furthermore, do the answers to the above questions obtained *in vitro* have any relevance to the function of the enzyme *in vivo*?
Biotin carboxylase is encoded by the accC gene, whereas BCCP is encoded by the accB gene. The two subunits involved in carboxyltransferase activity are encoded by the accA and accD genes. The covalently bound biotin of BCCP carries the carboxylate moiety.

The experimental approach for answering these questions was to make hybrid dimers of biotin carboxylase in which one subunit had a wild-type active site and the other subunit contained an active site mutation known to greatly decrease the activity of the enzyme (5, 10). The heterodimeric biotin carboxylase molecules were assayed for activity in vitro and also in vivo by measuring the rate of fatty acid biosynthesis in E. coli.

MATERIALS AND METHODS

Chemicals and Reagents—His-Bind resin used for the purification of proteins containing a poly-histidine tag was purchased from Novagen. FLAG octapeptide (NH2-DYKDDDDK-COOH) was synthesized at the protein synthesis facility at the University of Illinois. Anti-FLAG M2 antibody was obtained from Sigma. Collodion membranes used for the purpose of protein synthesis were from Biotin carboxylase is encoded by the accA gene, whereas BCCP is encoded by the accC gene. The duplicate gene construct was confirmed by restriction mapping.

Construction of Plasmids Encoding Tandem Flag-tagged Wild-type AccC Genes in Tandem with Various Flag-tagged Mutant AccC Genes—These constructions followed the general scheme described above. Plasmid pYML3 (Table I) was cut with EcoRI plus BamHI to obtain the E288K accC gene fragment, which was ligated to pMTL22p cut with the same enzymes to obtain plasmid pYML22. Plasmid pYML22 was cut with Ncol plus NdeI and ligated to the FLAG oligonucleotide containing the same ends, yielding plasmid pYML22-FLAG. The FLAG-tagged mutant accC gene was confirmed to introduce the FLAG-tagged wild-type accC gene plus the ribosome binding site, and this fragment was ligated to pMTL23p cut with the same enzymes, resulting in pYML23. Plasmid pYML23 was cut with HindIII plus XhoI releasing the FLAG-tagged gene and ligated to pGHC28 cut with the same enzymes, resulting in pWT/E288K.

Expression and Purification of Hybrid Biotin Carboxylases—For expression of the tagged proteins, the plasmids pWT/WT, pR92A/WT, pN290/WT, and pK238Q/WT were transformed into E. coli BLR(DE3) pLysS. A 100-fold dilution of an overnight saturated culture was made in 2× YT medium containing kanamycin and gentamicin. The culture was grown at 30 °C with constant agitation (300 rpm) until the A660 reached 0.6, and isopropyl-1-thio-β-D-galactoside was added to a final concentration of 2 mM to induce AccB protein expression. After 5 h of incubation the cells were harvested by centrifugation at 4 °C. The cell pellets were suspended in binding buffer (20 mM Tris-HCl, 500 mM NaCl, and 5 mM imidazole, pH 7.9) and lysed by three cycles of freezing and thawing the cell suspension (the phage T7 lysozyme encoded by pLysS makes the cells fragile to freeze-thaw treatment). DNase was added to degrade chromosomal DNA, and the lysate was centrifuged at 10,000 rpm for 4 °C for 1 h. The supernatant was supplemented with Triton X-100 (final concentration of 0.1%) and applied to a column containing immobilized nickel-chelate resin. The column was subsequently washed with 10 column volumes of binding buffer followed by 5 column volumes of binding buffer containing 60 mM imidazole. His-tagged biotin carboxylase was eluted from the column using binding buffer containing 250 mM imidazole. The purified protein ¹

¹ The abbreviations used are: WTBC, wild-type biotin carboxylase; CAPS, 3-(cyclohexylamino)propanesulfonic acid; WT, wild type.
was dialyzed against a solution of 0.67 mM EDTA and 10 mM potassium phosphate, pH 7.0, at 4 °C overnight.

For anti-FLAG M2 affinity chromatography, columns containing the anti-FLAG M2 affinity gel were washed with 0.1 mM glycine-HCl, pH 3.5, for 20 min and immediately equilibrated with Tris-buffered saline (50 mM Tris-HCl and 300 mM NaCl, pH 7.4). The protein from the His tag purification step was dialyzed against Tris-buffered saline and applied to the equilibrium columns. The columns were washed with 10 column volumes of Tris-buffered saline, and the bound protein was eluted with 20 ml of Tris-buffered saline containing 2 mg/ml FLAG tag. The fractions containing protein were pooled and dialyzed against 10 mM potassium HEPES, pH 7.0, containing 500 mM KC1 at 4 °C overnight. The protein solutions were concentrated by reverse osmosis using a collagen membrane with a 25,000 molecular weight cutoff. Protein concentrations were determined spectrophotometrically. The rate of ATP hydrolysis was calculated per active site using a molecular weight of 50,000 for the dimers.

Protein and Nucleic Acid Sequencing—Purified protein was transferred to nylon membrane using CAPS buffer, and N-terminal sequencing was done to confirm the presence of the N-terminal octapeptide FLAG tag fused to biotin carboxylase by the Protein Services Facility at the University of Illinois. All plasmid constructs were sequenced to confirm that the FLAG sequence had been added to the 5' end of the accC coding sequence. Both DNA and N-terminal protein sequencing were done by the University of Illinois Biotechnology Center.

Biocytin Carboxylase Assay—The activity of biocytin carboxylase was determined spectrophotometrically. The rate of ATP hydrolysis was measured by detecting the production of ADP using pyruvate kinase and lactate dehydrogenase and following the oxidation of NADH at 340 nm. Each reaction contained 17.5 units of lactate dehydrogenase, 10.5 units of pyruvate kinase, 0.5 mM phosphoenolpyruvate, 0.2 mM NADH, 8 mM MgCl2, 15 mM potassium bicarbonate, and 100 mM HEPES at pH 8.0. All reactions were carried out in a total volume of 0.5 ml in a 1-cm path-length quartz cuvette. When the kinetic parameters for biocytin were determined, ATP was added to the reaction and held constant at 150 mM. All reactions were initiated by the addition of enzyme and were maintained at 25 °C via a circulating water bath. All spectrophotometric data was collected using a Uvikon 300 (Kontron Instruments) spectrophotometer interfaced to a PC with a data acquisition program.

Data Analysis—The kinetic parameters $K_m$ and $V_{max}$ were determined by fitting the initial velocities versus the substrate concentration to the Michaelis-Menten equation using nonlinear regression analysis performed by the program Enzfitter. Values for $V_{max}$ and $K_m$ were calculated per active site using a molecular weight of 50,000 for the biocytin carboxylase monomer.

RESULTS AND DISCUSSION

Preparation and Isolation of Hybrid Biotin Carboxylase Dimers—Hybrid biocytin carboxylase dimers were made in vivo
by co-expression of differently tagged accC genes arranged in tandem as a synthetic operon (Fig. 2). Both modified accC coding sequences had 5' extensions to the coding sequence that encoded tags for affinity purification of the encoded AccC protein. One of the accC genes was marked with a His tag, and the other accC gene was marked with a FLAG tag. The sequences encoding the affinity tags were fused to the N terminus of biotin carboxylase instead of the C terminus, because the N-terminal location does not compromise the activity of the enzyme (5). A gene cassette encoding gentamicin resistance was placed between the two accC genes to preclude the possibility of genetic recombination between the two genes, which could invalidate the experiments. Homologous recombination between the two copies would delete the antibiotic resistance cassette, resulting in sensitivity to gentamicin and inhibition of protein synthesis and subsequent cell death.

When the genes for differently tagged biotin carboxylase were co-expressed from a plasmid (Fig. 2), three different types of biotin carboxylase dimers were formed in vivo (Fig. 3). One type of dimer had two His-tagged subunits (called HH). Both subunits of another dimer type were FLAG-tagged (FF), and the third dimer had one subunit with a His tag and the other subunit with a FLAG tag (HF). There also is the possibility of formation of small amounts of a dimer containing a chromosomally encoded wild-type (WT) untagged subunit plus either a His- or FLAG-tagged subunit. The crude *E. coli* soluble extract was first eluted from an immobilized nickel affinity column that bound all His tag-containing biotin carboxylase dimers (i.e. HH and HF), and eliminated the FF dimer (Fig. 3). The mixture of eluted HH and HF dimers were then chromatographed on the anti-FLAG affinity column to eliminate the HH dimer. The HF dimer was then eluted from the column. N-terminal sequencing was carried out to confirm that the purification protocol (Fig. 3) did produce the HF biotin carboxylase hybrid. The results showed the presence of both the His-and FLAG-tag sequences in the N terminus of the purified protein (data not shown). Note that our preparation procedure also removes any dimers that contained the wild-type (WT) subunit encoded by the chromosomal accC gene.

It should be noted that the purification method would be compromised if a rapid equilibration occurred between the monomer and dimer forms of the protein. For example, if the intent was to purify dimers having one His-tagged normal and one FLAG-tagged mutant subunit (an HF dimer) and rapid subunit exchange did occur, then the HF dimer would rearrange to give rise to HH and FF dimers, which would complicate the analyses. In the case of biotin carboxylase this seemed unlikely to be a problem, because the monomeric protein has not been observed unless denaturing conditions are used. However, we tested for subunit exchange by mixing a culture expressing only a His-tagged biotin carboxylase with an otherwise identical culture that expressed only a FLAG-tagged biotin carboxylase. The cell-free extract of the mixed culture was then chromatographed as above. If subunit exchange occurred, then a protein species that bound to both column matrices would be formed. We were unable to detect formation of

![Fig. 2. Construction of a plasmid for expression of a differently tagged biotin carboxylase. FWTBC, the gene encoding FLAG-tagged wild-type biotin carboxylase; HWTBC, the gene encoding His-tagged wild-type biotin carboxylase.](http://www.jbc.org/doi/abs/10.1074/jbc.M111.274883)
such a species. These results were confirmed by the analyses reported below, because appreciable subunit exchange would have resulted in appreciable levels of carboxylase activity caused by the formation of active dimers from dimers that contained one active subunit and one inactive subunit.

The experimental approach described here for the preparation and purification of hybrids of biotin carboxylase is generally applicable to any dimeric protein. Moreover, this method offers several advantages over previous approaches for making hybrids of oligomeric proteins (11–13). For instance, earlier preparations of hybrids of oligomeric proteins were done in vitro and involved dissociation of the subunits, sometimes by complete denaturation, followed by mixing with the mutated subunits to form hybrids plus parental molecules. Separation of the hybrid proteins from the parental molecules relied upon a difference in charge between the mutant and wild-type subunits. If the mutation produced no change in charge between the hybrid and parental proteins then a charge was introduced into one of the subunits by chemical modification, usually during the denaturation step. Such chemical modifications may or may not alter the activity of an enzyme. In contrast, the method described in this paper avoids dissociation and denaturation of the protein by allowing hybrids to form in vitro. Moreover, the hybrids were purified by affinity chromatography rather than by altering the hybrid by a potentially deleterious chemical modification.

Note that our experimental approach is also of advantage when a mutant protein is expressed in its natural (or a closely related host), and the wild-type protein is essential for growth (as is the case for accC). The indispensability of the protein means that the host gene cannot be deleted, and thus the preparations of the mutant protein will be contaminated with the wild-type protein encoded by the chromosomal gene. (This is the result of formation of dimers containing one affinity-tagged mutant subunit and an untagged wild-type subunit.) Such contamination can be minimized by high levels of expression of the mutant protein and/or by use of an affinity tag fused to the mutant protein, but even when both strategies are used some wild-type protein will be present. However, the use of differently tagged genes expressed in tandem as described here followed by two affinity columns eliminates the possibility of contamination by the wild-type chromosomally encoded protein.

**In Vitro Analysis of Biotin Carboxylase Hybrids**—Four different His-tagged and FLAG-tagged (HF) biotin carboxylase hybrid dimers were prepared. In each hybrid dimer one subunit had the wild-type active site, and the second subunit contained a mutation of an active site residue. The hybrids were characterized kinetically by determining the maximal velocity ($V_{\text{max}}$) for the rate of biotin carboxylation as well as the Michaelis constant ($K_m$) for biotin. The $V_{\text{max}}$ and $K_m$ values for the hybrids were compared with the values for HF biotin carboxylase, in which both subunits have the wild-type active site. The results for the four hybrids and wild-type control are given in Table II along with previously determined $V_{\text{max}}$ and $K_m$ values for the mutant and wild-type enzymes, in which both subunits carried the His tag. Comparison of the $V_{\text{max}}$ and $K_m$ values for the HF biotin carboxylase having a wild-type active site with that of the HH wild-type active site enzyme show that the presence of the FLAG tag on the N terminus did not effect the activity of the enzyme. A previous study determined that His-tagged wild-type enzyme has the same activity as the native biotin carboxylase (5). Thus, any effects observed in the HF biotin carboxylase hybrid dimers were caused by the mutant subunit rather than the presence of the N-terminal tags.

The mutations (R292A, N290A, K238Q, and E288K) used to make the four hybrids of biotin carboxylase were chosen for two reasons. First, all four mutations are located within the active site of the enzyme, which from structural studies is known to be far removed from the interface of the two subunits. Therefore, any observed kinetic effect on the wild-type subunit of the hybrid would be caused by long-range communication between the active sites. Second, when both subunits were mutant, three of the mutations (R292A, N290A, and K238Q) resulted in an ~200-fold decrease in the $V_{\text{max}}$ of biotin carboxylase, whereas the E288K mutation gave a completely inactive enzyme (Table II). Kinetic analyses of hybrid biotin carboxylase dimers, in which one subunit contained a wild-type active site and the other subunit contained one of the four active site mutations, showed that the $V_{\text{max}}$ values of the four hybrid dimers were 0.4–3.6% of the $V_{\text{max}}$ value for the HF wild-type active site dimer (Table II). For three of the hybrid dimers, R292A/WT, N290A/WT, and K238Q/WT, the initial velocity was independent of the concentration of biotin. Therefore, $K_m$ values for biotin could not be determined, and the $V_{\text{max}}$ values given in Table II for these three dimers are the average initial velocities. For the K238Q/WT hybrid dimer, the initial velocity depended on the biotin concentration, allowing the Michaelis constant for biotin to be determined. In contrast, when both subunits of biotin carboxylase contained the K238Q mutation,
Subunit Interaction in Biotin Carboxylase

Kinetic parameters for wild-type and mutant heterodimeric biotin carboxylases

| Enzyme<sup>a</sup> | \( V_{\text{max}} \) <sup>b</sup> | \( K_{\text{m}} \) <sup>c</sup> |
|-------------------|------------------|------------------|
| WT/WT (HH)        | 78.6 ± 3.2       | 134.0 ± 13.8     |
| WT/WT (HF)        | 99.5 ± 19.9      | 192.7 ± 42.2     |
| R292A/R292A (HH)  | 0.39 ± 0.02      | 123.6 ± 14.2     |
| R292A/WT (HF)     | 2.54 ± 1.30      | —                |
| N290A/N290A (HH)  | 0.31 ± 0.02      | 60.1 ± 8.8       |
| N290A/WT (HF)     | 3.60 ± 1.01      | —                |
| K238Q/K238Q (HH)  | 0.43 ± 0.01      | —                |
| K238Q/WT (HF)     | 1.06 ± 0.04      | 64 ± 7           |
| E288K/E288K (HH)  | 0.005            | —                |
| WT/E288K (HF)     | 0.35 ± 0.03      | —                |

<sup>a</sup> Nomenclature used: HH denotes the form of biotin carboxylase in which both subunits contain an N-terminal His tag. HF denotes the differentially tagged form of biotin carboxylase in which one subunit contains an N-terminal His tag, and the other contains an N-terminal FLAG tag.

<sup>b</sup> The kinetic parameters were determined by varying the concentrations of biotin at constant saturating levels of bicarbonate and ATP. The values reported for WT and mutant differentially tagged biotin carboxylases are the average of replicate measurements ± the standard error.

<sup>c</sup> Data are taken from Ref. 5. The activity listed for E288K/E288K (HH) is equivalent to the background activity (i.e. the rate of ATP hydrolysis in the absence of protein).

<sup>d</sup> For these mutant differentially tagged biotin carboxylases, both the \( V_{\text{max}} \) and \( K_{\text{m}} \) could not be calculated by fitting their initial velocities to the Michaelis-Menten equation; therefore, the values listed for \( V_{\text{max}} \) are actually the approximate values for \( V_{\text{max}} \) based on their initial velocities at a particular biotin concentration.

<sup>e</sup> Data are taken from Refs. 10 and 7, respectively. The activity listed for E288K/E288K (HH) is equivalent to the background activity (i.e. the rate of ATP hydrolysis in the absence of protein).

<sup>f</sup> A \( K_{\text{m}} \) value could not be determined because the activity of the enzyme was independent of the biotin concentration.

In Vivo Analysis of Biotin Carboxylase Hybrids—The in vitro results predicted that the active site mutants should act as dominant negative mutations when sufficient mutant protein was produced in vivo. That is, in the presence of excess mutant monomer most of the wild-type monomer should be sequestered into hybrid dimers, which would have severely reduced activities.

To assess the in vivo activity of the four hybrids of biotin carboxylase, the rate of fatty acid biosynthesis in E. coli was determined. The genes coding for each of the four biotin carboxylase mutants were cloned into the expression vector pBAD30, which is a medium copy-number plasmid. The rationale of this approach was to produce a sufficient concentration of mutant subunits to combine with the wild-type subunit encoded by the E. coli chromosome and sequester the wild-type subunits in hybrid dimers. The rate of fatty acid biosynthesis was determined by measuring the incorporation of [1-14C]acetate into cellular phospholipid (Table III). The rate of fatty acid synthesis was the same in the presence of the vector pBAD30 or the plasmid containing the gene for a wild-type subunit of biotin carboxylase. In contrast, when the vector coded for one of the four mutants of biotin carboxylase, the extent of fatty acid biosynthesis was decreased by an order of magnitude. These results are consistent with the in vitro data and suggest that the hybrids are exhibiting a dominant negative effect in vivo as well.

Conclusions—There are numerous examples of long-range communication in proteins. For example, when a ligand or substrate binds to a receptor or enzyme, there is often a subsequent conformational change in the protein. Allosteric enzymes as effector molecule can bind to one subunit, causing a change in the affinity for substrate that binds to a different subunit. In these instances, the communication between subunits is manifested in the normal function of the protein. However, in the case of biotin carboxylase, there is no apparent communication between the two subunits (e.g. cooperative ki-
netics) during the normal function of the enzyme. In other words, the two subunits seem to function completely independently. Our studies, however, demonstrate very clearly that there is communication between the two subunits of biotin carboxylase. The difference between the work described in this paper and the examples mentioned above is that the communication is manifested only when one of the subunits contains a mutation that affects enzymatic activity. The only other example of cooperativity in biotin carboxylase also comes from the study of mutant enzymes. The N290A mutant of biotin carboxylase exhibited negative cooperativity with respect to the substrate carbamyl phosphate in the model reaction catalyzed by biotin carboxylase, in which carbamyl phosphate reacts with ADP to make ATP (5). Further studies will be required to determine the detailed molecular mechanism by which a mutation in the active site of one biotin carboxylase subunit is communicated to the other subunit. However, we envision a possible starting point for a molecular model that may explain the dominant negative effect. The two subunits of biotin carboxylase may be unable to catalyze the reaction simultaneously. Instead, the two subunits must alternate catalytic reactions such that while one subunit is binding substrate and undergoing catalysis, the other subunit is releasing product. If one of the subunits has a mutation resulting in a significant decrease in catalytic rate, this would also lead to a decrease in the catalytic rate of the wild-type subunit, because the catalytic sequences of the two subunits would be mechanistically linked in a fixed cycle.

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*J. Biol. Chem.* 2001, 276:29864-29870.
doi: 10.1074/jbc.M104102200 originally published online June 4, 2001

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