The *Escherichia coli* Cytochrome c Maturation (Ccm) System Does Not Detectably Attach Heme to Single Cysteine Variants of an Apocytochrome c*

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Cytochromes *c* are typically characterized by the covalent attachment of heme to polypeptide through two thioether bonds with the cysteine residues of a Cys-Xaa-Xaa-Cys-His peptide motif. In many Gram-negative bacteria, the heme is attached to the polypeptide by the periplasmically functioning cytochrome *c* maturation (Ccm) proteins. Exceptionally, *Hydrogenobacter thermophilus* cytochrome *c*₅₅₂, which has a normal CXCH heme-binding motif, and variants with AXCH, CXXAH, and AXXAH motifs, can be expressed as stable holocytochromes in the cytoplasm of *Escherichia coli*. By targeting these proteins to the periplasm using a signal peptide, with or without co-expression of the Ccm proteins, we have assessed the ability of the Ccm system to attach heme to proteins with no, one, or two cysteine residues in the heme-binding motif. Only the wild-type protein, with two cysteines, was effectively processed and thus accumulated in the periplasm as a holocytochrome. This is strong evidence for disulfide bond formation involving the two cysteine residues of apocytochrome *c* as an intermediate in Ccm-type Gram-negative bacterial cytochrome *c* biogenesis and/or that only a pair of cysteines can be recognized by the heme attachment apparatus.

**It is commonly assumed, but it has not been proven, that formation of a disulfide bond between the two cysteine residues of apocytochrome *c* is an intermediate in bacterial cytochrome *c* biogenesis by the Ccm system. The cysteine thiols are thought to be oxidized to a disulfide by DsbA, one of the proteins of the Dsb disulfide isomerase system, which actively forms disulfide bonds in the periplasm (2, 13). We have recently shown that a bacterial apocytochrome *c* can spontaneously form an intramolecular disulfide bond in oxidizing conditions even in the absence of DsbA (14). It would be necessary for any intramolecular disulfide in apocytochrome *c* to be reduced before the thiols can react with the vinyl grops of heme. CcmG and CcmH both contain the thioredoxin motif (CXXC) of disulfide reductases (13); at least one of them has been argued to reduce the cysteines of apocytochrome *c* before heme attachment, ultimately by transferring electrons from DsbD (also known as DipZ) (13, 15). DsbD receives electrons from the cytoplasmic TrxA and transfers them to the periplasm. Mutants of *E. coli* deficient in DsbA, DsbB (the oxidant for DsbA), DsbD, or TrxA were all unable to synthesize *c*-type cytochromes (16–18). However, it is not clear whether this was because of the inability of these mutants to process a disulfide involving apocytochrome *c*, one or more of the Ccm proteins, or indeed a combination of these proteins. The importance of investigating whether the substrate, *i.e.*, cytochrome *c*, or the biogenesis apparatus requires disulfide bonds is emphasized by work on the type II secretion system in Gram-negative bacteria (19). The requirement for DsbA was initially taken to mean that a secreted protein needed a disulfide bond; later it was shown that the disulfide was required in the biogenesis machinery and that a modified substrate could be secreted with a single cysteine thiol instead of a disulfide between two cysteines (19).

It is important for understanding the operation of the Ccm system to determine whether it can assemble cytochromes *c* in which the heme is attached to the apoprotein by only one thioether bond. Among other things, this would provide strong evidence as to whether an intramolecular disulfide bond in an apocytochrome *c* is indeed an intermediate. Rıos-Velázquez *et al.* (20) made single and double cysteine to alanine substitutions in the heme-binding motif of *Rhodobacter sphaeroides* cytochrome *c*₂. Such variant proteins were unable to support growth via photosynthesis where functional cytochrome *c*₂ is required. However, in their experiments, no product cytochromes with these substitutions, either holo or apo forms, could be isolated. This indicates that the proteins produced were unstable and rapidly degraded *in vivo*; thus it is not possible to say definitely whether or not the heme was covalently attached to the polypeptides by the Ccm proteins of *R. sphaeroides* before degradation occurred. Sambongi *et al.* (21) conducted similar experiments using heme-binding motif variants of *Paracoccus denitrificans* cytochrome *c*₅₅₀, but again the

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*This work was supported by Biotechnology and Biological Sciences Research Council Grant C13443 (to S. J. F.). 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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The abbreviation used is: Ccm, cytochrome *c* maturation.
products were unstable. Unusually heme may be covalently (and correctly) attached to cytochrome \( c_{552} \) from *Hydrogenobacter thermophilus* in the cytoplasm of *E. coli* apparently without the action of any specialized biosynthesis proteins (22–24). Replacement of one or both of the heme-binding cysteine residues by alanines (C11A, C14A, and C11A/C14A) also results in formation of stable cytochromes (25, 26). In the two former cases, the heme is covalently attached to the polypeptide through a single thioether bond; in the latter the product is a b-type cytochrome whose heme is noncovalently bound to the polypeptide. Therefore, we have used *H. thermophilus* cytochrome \( c_{552} \) and the C11A, C14A, and C11A/C14A variants to investigate the functioning of the Ccm system. Each of the proteins has been targeted to the periplasm of *E. coli* where the Ccm proteins act and expressed with or without plasmid-borne *ccm* genes.

**Experimental Procedures**

*E. coli* strain JCB387 (27) was transformed with an appropriate plasmid encoding for *H. thermophilus* cytochrome \( c_{552} \), C11A, C14A, or C11A/C14A variants. Two forms of these plasmids were used; plasmids pHKH12 (22), pEST201, pEST202, and pEST203 (25, 26) with no periplasmic signal sequence, resulting in cytoplasmic expression of the cytochromes, have been described previously. New plasmids were constructed with the signal sequence of *Pseudomonas aeruginosa* cytochrome \( c_{552} \) to target the apocytochromes to the periplasm. PCR was used to amplify and fuse the signal sequence gene to the cytochrome gene on plasmids pHKH12, pEST201, pEST202, and pEST203 using the same method as Zhang et al. (28). pEST210, which carries wild-type cytochrome \( c_{552} \) and the signal sequence, was the kind gift of Dr. Y. Sambongi. Each of these new plasmids was sequenced to ensure that the signal sequence was present and that there were no secondary mutations in the cytochrome gene. Cells could also be co-transformed with pEC86 (29), which carries the *E. coli* cytochrome \( c \) maturation genes *ccmAABCDEFOGH*. *E. coli* cytochrome \( b_{562} \) with a periplasmic targeting sequence was produced using a plasmid described previously (30); \( b_{562} \) cell cultures were grown identically to those transformed with the other plasmids, and the plasmid-borne *ccm* genes were co-transformed as required.

Cells were initially grown on LB-agar plates with the appropriate antibiotics (100 \( \mu \)g ml\(^{-1}\) ampicillin in each case plus 54 \( \mu \)g ml\(^{-1}\) chloramphenicol where pEC86 was co-transformed). Single colonies were picked into 500-ml 2× TY medium (16 g liter\(^{-1}\) peptone, 10 g liter\(^{-1}\) yeast extract, 5 g liter\(^{-1}\) NaCl) supplemented with 1 mM isopropyl-

**Results**

*E. coli* strain JCB387 was transformed with various plasmids to assess the ability of the *E. coli* cytochrome \( c \) maturation proteins to process wild-type *H. thermophilus* cytochrome \( c_{552} \) and C11A, C14A, and C11A/C14A variants; these have CXXCH, AXXCH, CXXAH, and AXXAH heme-binding motifs, respectively. Each of these four proteins was available with or without a periplasmic targeting sequence. The latter enables translocation of the polypeptide to the periplasm where the Ccm system operates. Thus, in our investigations, 16 combinations were used: cytochrome \( c_{552} \), C11A, C14A, and C11A/C14A \( \pm \) a periplasmic signal sequence, each \( \pm \) the plasmid-borne *ccm* genes. *E. coli* strain JCB387 (27) was chosen because preliminary experiments indicated that it produced only moderate yields of each of the four *H. thermophilus* cytochromes cytoplasmically (i.e. in the absence of the targeting sequence the cytochrome production was detectable but not as such as to swamp any periplasmic cytochromes that may be produced with a targeting sequence). Strain JCB387 also produced a poor yield of periplasmically targeted wild-type cytochrome \( c_{552} \) in the absence of the co-transformed *ccm* plasmid. Cells were grown aerobically, so that expression of the endogenous Ccm system of *E. coli* would be minimized, and were fractionated into periplasmic and cytoplasmic components; each fraction was assayed for contamination by the other using enzyme marker assays. The fractionation protocol was carefully optimized to result in minimal contamination of the periplasmic fraction with cytoplasmic proteins and vice versa (see “Experimental Procedures”). Quantities of cytochrome produced were determined spectrophotometrically.

In the absence of a periplasmic signal sequence, effectively 100% of each of the cytochromes was made cytoplasmically (Table I).\(^2\) These data were as anticipated and serve both as a control experiment and as a reference against which expression with a periplasmic signal sequence can be assessed. In the presence of the signal sequence, of cytochrome \( c_{552} \) and each of the three variants studied, only the wild-type holoprotein was found in significant quantities in the periplasm. With the signal sequence and the *ccm* plasmid present, essentially 100% of the wild-type cytochrome was periplasmic (Table II). In contrast, the variant cytochromes were found in the periplasm only in small amounts even when the Ccm proteins were co-expressed (in each case less than 5% of the total cytochrome after subtraction for cytoplasmic contamination and endogenous cytochrome production by *E. coli* (see below)). In the presence of the periplasmic targeting sequence, with or without

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\(^2\) Note that subtraction from the percentage of the total cytochrome in the periplasmic fraction (Tables I and II, fourth column) is necessary to correct for contamination of the periplasmic fraction by cytoplasmic proteins as indicated by malate dehydrogenase assays (Tables I and II, fifth column).
The periplasmic and cytoplasmic marker proteins were co-transformed with the plasmid carrying the cytochrome gene. The periplasmic and cytoplasmic marker proteins were \( \beta \)-lactamase and malate dehydrogenase, respectively. The percentage of the total cytochrome in the periplasm and the yield of total cytochrome per gram of wet cells are corrected from the raw measurements by subtracting a constant background level of endogenous \( E. \) coli cytochromes (see "Results").

### Table I

**Distribution in \( E. \) coli of \( H. \) thermophilus cytochrome \( c_{552} \) and variants with altered heme-binding motifs expressed from genes lacking signal sequences**

| Heme-binding motif | Presence of the \( ccm \) plasmid | % of the periplasmic marker protein in the periplasmic fraction | % of the total \( H. \) thermophilus cytochrome in the periplasmic fraction | % of the cytoplasmic marker protein in the periplasmic fraction | Total \( H. \) thermophilus cytochrome (mg/g wet cells) |
|-------------------|-----------------------------------|-------------------------------------------------------------|------------------------------------------------------------------|-------------------------------------------------------------|-------------------------------------------------|
| CXCH              | -                                 | 79                                                          | 10                                                               | 19                                                          | 0.9                                            |
| CXCH              | +                                 | 76                                                          | 7                                                                | 5                                                            | 1.0                                            |
| AXCH              | -                                 | 70                                                          | 7                                                                | 19                                                          | 1.2                                            |
| AXCH              | +                                 | 92                                                          | 3                                                                | 3                                                            | 1.5                                            |
| CXXAH             | -                                 | 74                                                          | 17                                                               | 19                                                          | 0.44                                           |
| CXXAH             | +                                 | 74                                                          | 5                                                                | 8                                                            | 0.96                                           |
| AXAX              | -                                 | 89                                                          | 1                                                                | 11                                                          | 1.8                                            |
| AXAX              | +                                 | 85                                                          | 3                                                                | 2                                                            | 2.2                                            |

These control data are the averages of two or three experiments. \( Ccm \) + indicates that the plasmid-borne cytochrome \( c \) maturation genes were co-transformed with the plasmid carrying the cytochrome gene. The data are presented as the mean of multiple experiments; standard deviations are shown in parentheses. The periplasmic and cytoplasmic marker proteins were \( \beta \)-lactamase and malate dehydrogenase, respectively. The percentage of the total cytochrome in the periplasm and the yield of total cytochrome per gram of wet cells are corrected from the raw measurements by subtracting a constant background level of endogenous \( E. \) coli cytochromes (see "Results").

### Table II

**Distribution in \( E. \) coli of \( H. \) thermophilus cytochrome \( c_{552} \) variants with altered heme-binding motifs, and \( E. \) coli cytochrome \( b_{562} \) expressed from genes with signal sequences**

| Heme-binding motif | Presence of the \( ccm \) plasmid | % of the periplasmic marker protein in the periplasmic fraction | % of the total \( H. \) thermophilus cytochrome (or of cyt. \( b_{562} \)) in the periplasmic fraction | % of the cytoplasmic marker protein in the periplasmic fraction | Total \( H. \) thermophilus cytochrome (or cyt. \( b_{562} \)) (mg/g wet cells) |
|-------------------|-----------------------------------|-------------------------------------------------------------|------------------------------------------------------------------|-------------------------------------------------------------|-------------------------------------------------|
| CXCH              | -                                 | 68 (17)                                                     | 40 (11)                                                          | 4 (2)                                                       | 0.66 (0.26)                                    |
| CXCH              | +                                 | 79 (12)                                                     | 82 (10)                                                          | 3 (0)                                                       | 3.0 (1.5)                                      |
| AXCH              | -                                 | 63 (12)                                                     | 13 (5)                                                           | 16 (11)                                                    | 0.50 (0.28)                                    |
| AXCH              | +                                 | 80 (15)                                                     | 4 (6)                                                            | 5 (4)                                                       | 0.73 (0.38)                                    |
| CXXAH             | -                                 | 72 (16)                                                     | 0 (1)                                                            | 6 (3)                                                       | 0.51 (0.28)                                    |
| CXXAH             | +                                 | 79 (7)                                                      | 13 (5)                                                           | 8 (7)                                                       | 0.51 (0.11)                                    |
| AXAX              | -                                 | 80 (5)                                                      | 3 (3)                                                            | 10 (3)                                                      | 0.49 (0.12)                                    |
| AXAX              | +                                 | 85 (10)                                                     | 9 (4)                                                            | 6 (5)                                                       | 0.93 (0.48)                                    |
| RXYH in cyt. \( b_{562} \) | -                                 | 83                                                          | 85                                                               | 20                                                          | 5.4                                            |
| RXYH in cyt. \( b_{562} \) | +                                 | 93                                                          | 84                                                               | 12                                                          | 4.1                                            |

When wild-type cytochrome \( c_{552} \) was expressed with the periplasmic signal sequence but no \( ccm \) plasmid, a significant fraction of the \( c_{552} \) was found in the periplasmic fraction (on the \( ccm \) plasmid, there was no significant quantitative difference in the total expression level or periplasmic quantities of the \( AXXAH, CXXAH, \) or \( AXCH \) proteins (Table II). Small quantities of variant proteins in the periplasm can be accounted for by self-assembly; if protein with a signal sequence is translocated to the periplasm and encounters heme, it can be expected to spontaneously form a cytochrome as it does in the cytoplasm. Within experimental error, targeting the apocytochromes to the periplasm caused a decrease in total holocytochrome production in every case except for the wild-type cytochrome \( c_{552} \) when co-expressed with the Ccm proteins (compare data in Table II with data in Table I). Moreover, the results in Table II (and described above) show that the vast majority of the periplasmically targeted holocytochromes with substitutions in the heme-binding motif were made cytoplasmically despite the presence of the signal sequence. Staining SDS-polyacrylamide gels for covalently bound heme for such periplasmically targeted \( c_{552} \) variants resulted in a band at a higher molecular weight than for cytoplasmically produced protein with no targeting sequence, reflecting the presence of both heme and the targeting sequence in the higher molecular weight material. It might be anticipated that the availability of the periplasmic signal sequence would cause rapid translocation of the apoprotein before heme attachment could occur in the cytoplasm. However, our data indicate that there is competition between the rate of heme binding to apoprotein in the cytoplasm and the rate of apoprotein translocation to the periplasm by the general type II secretion (Sec) proteins. Translocation may be slowed because \( H. \) thermophilus apocytochromes \( c_{552} \) are quite structured (36), and the Sec system transports unfolded proteins (37). The observation that the periplasmically targeted double alanine (C11A/C14A) variant protein, which forms a \( b \)-type cytochrome (25), was not made in significant quantities in the periplasm, either with or without the Ccm system, is an apparent anomaly. In our experimental conditions, \( E. \) coli cytochrome \( b_{562} \) with a periplasmic signal sequence was made effectively 100\% periplasmically with or without co-expression of the \( ccm \) plasmid (Table II). It is well established that disruption of the Ccm proteins does not inhibit \( b_{562} \) formation in the periplasm of \( E. \) coli (38, 39); hence we anticipated that C11A/C14A \( c_{552} \) would be made equally well. One plausible explanation for our observations is that, when in the periplasm, the apo form of the C11A/C14A protein, which is not a naturally occurring \( b \)-type cytochrome, acquires its heme less quickly than and/or is more susceptible to proteolysis than apocytochrome \( b_{562} \). This would not affect the wild-type cytochrome \( c_{552} \), which can rapidly acquire heme from the Ccm system. The means by which \( b \)-type cytochromes acquire heme in the bacterial periplasm, a compartment of the cell in which they are rare, is far from understood, so the specific nature of the apocytochrome may, therefore, also be important for the process.

When wild-type cytochrome \( c_{552} \) was expressed with the periplasmic signal sequence but no \( ccm \) plasmid, a significant fraction of the \( c_{552} \) was found in the periplasmic fraction (on
average ~60%). This suggests that the endogenous E. coli Ccm system, which is maximally expressed under anaerobic conditions (40), was being expressed to some extent during growth (i.e. our cultures were not fully aerobic). However, even with this level of expression of periplasmic cytochrome c552, the yield in mg of periplasmic cytochrome produced per g of wet cells was only ~14% of that produced when the ccm plasmid was present; thus this effect is relatively minor. As a corollary, wild-type c552 with a periplasmic signal sequence but no ccm plasmid produced ~40% cytoplasmic cytochrome c, whereas in the equivalent case with the ccm plasmid, the latter value was ~0%. Furthermore, any low level expression of the cells' own Ccm proteins has not affected our data for the cytochromes with substitutions in the heme-binding motif since the introduction of the ccm plasmid (which implies a much higher level of expression) makes no significant quantitative difference to the periplasmic cytochrome expression levels of these variant proteins.

To determine background absorbances in our measurements, we assessed the level of endogenous cytochrome production by E. coli strain JCB387 not transformed with any of our plasmids. In the periplasmic fractions of such cells, we detected a c-type cytochrome ($\lambda_{\text{max}}$ 551 and 419 nm), probably NapB, a soluble subunit of the periplasmic nitrate reductase (41). In the cytoplasm we detected absorbance from one or more hemo­proteins (observed $\lambda_{\text{max}}$ ~560 and ~423 nm), e.g. catalase. The periplasmic cytochrome accounted for 33% of the total absorbance, and the cytoplasmic cytochrome(s) accounted for 67% with the periplasmic and cytoplasmic fractions normalized for volume. These proportions were essentially the same (32 and 68%) if E. coli JCB387 was transformed with the ccm plasmid pEC86 but no exogenous cytochrome plasmid. One might expect that expression of the Ccm proteins from the plasmid would stimulate production of the endogenous periplasmic c-type cytochrome. However, the nap and ccm operons are co-regulated in E. coli (11, 42), so in a given set of growth conditions, the expression of NapB would be limited by the same factor(s) with or without co-expression of the Ccm proteins from pEC86. We have corrected for these background hemoprotein absorbances in Tables I and II assuming that the endogenous cytochromes were produced at the same levels per gram of wet cells in all of our growth experiments; the effect is to reinforce the conclusions drawn in the paragraphs above.

The percentages of each of the variant cytochromes (A XX to reinforce the conclusions drawn in the paragraphs above.

endogenous cytochromes were produced at the same levels per
hemoprotein absorbances in Tables I and II assuming that the
c馕 corrected for these background
descriptions were in fact due to
endogenous cytochrome(s).

DISCUSSION

The principal result of this study is that the type I cytochrome c maturation system as found in many Gram-negative bacteria can only effectively process covalent heme attachment to apocytochrome c with two cysteine residues in the heme-binding motif. The nature of our experiments is such that we cannot say with certainty that the activity of the Ccm system toward the substrate apoproteins with single or no cysteine heme-binding motifs is 0%, rather than <2%, of that of the double cysteine apocytochrome; nevertheless, the latter value is a reasonable estimate of the upper limit of any such activity. The implication is that an intramolecular disulfide bond within apocytochrome c is, as postulated on the basis of less direct evidence, an intermediate in this type of cytochrome c biogenesis. This idea finds support in the observation that H. thermophilus apocytochrome c552 forms an intramolecular disulfide bond under oxidizing conditions following removal of the heme in vitro (14). An alternative explanation for the data presented in the present work is that that both apocytochrome cysteine thiol s play specific, and required, roles in the maturation pathway, possibly involving intermolecular disulfide bonds. A further interpretation, which may or may not be combined with the obligate formation of one or more disulfide bonds, is that the ultimate highly specific recognition determinant for heme attachment by the Ccm system is the two cysteine thiols in the apocytochrome heme-binding motif. Note, however, that any rationalization of our data must allow for the fact that the Ccm proteins are active with substrate apocytochromes that either have naturally (43), or have as the result of amino acid substitutions (20), CXXCH or CXXCH heme binding-motifs, and thus it is the two cysteines that are important rather than their precise spatial arrangement.

It is very possible that the CXXCH motif of apocytochrome c cannot avoid being oxidized to a disulfide in the bacterial periplasm, e.g. by the active oxidant DsbA, and thus that the Ccm system has evolved to handle such oxidized apoproteins. Indeed it has been suggested (13) that formation of a disulfide in the apocytochrome may pre-fold the polypeptide and that this facilitates covalent heme attachment. The likelihood that a disulfide bond is an intermediate in holocytochrome c formation also implies that covalent heme attachment, or formation of a mixed disulfide between one of the apocytochrome cysteines and a cysteine from one of the Ccm proteins, is concerted with reduction of the apocytochrome disulfide. Otherwise the reduced disulfide is susceptible to rapid reoxidation by, for example, DsbA.

The crucial difference between our failure to observe covalent attachment of heme to apocytochromes with a CXXAH or AXXCH heme-binding motif and previous related studies (20, 21) is that in the earlier cases the products were unstable with respect to degradation. Thus is was not possible to determine unequivocally whether heme was in fact covalently attached by the bacterial Ccm system before the protein degraded. Moreover, there is no evidence that single cysteine proteins with covalently bound heme could ever actually form from the variant apocytochromes tested in the earlier studies. In the present work, we have used H. thermophilus cytochrome c552 together with mutants that are known to form holocytochromes and to be stable when expressed in the cytoplasm of E. coli (Table I and Refs. 25 and 26); the wild-type cytochrome is clearly also stable in the periplasm (Table II). Thus, our inability to observe variant holocytochromes in significant quantities in the periplasm is convincing evidence that the Ccm system cannot covalently attach heme to apocytochromes that do not have two cysteine residues in the heme-binding motif. Previous studies have shown that apocytochrome c with a periplasmic signal sequence, even with substitutions in the heme-binding motif, is translocated into the periplasm independent of the heme attachment process (21). Single cysteine-attached cytochromes (XXXXCH motif) have been isolated from some eukaryotic sources (44–47). In two cases (46, 47) the formation of these cytochromes was catalyzed by a specific enzyme, cytochrome c heme lyase from yeast mitochondria, but the yields were low relative to protein with a CXXCH heme-binding motif. It may be that other mitochondrial heme lyases have evolved where necessary to cope with only having one thiol for heme attachment. No single cysteine-attached cytochromes c have been observed to date in bacteria, which is consistent with the inability of the Ccm system, one of two known bacterial cytochrome c biogenesis systems (1, 2, 10), to process them. Parts of the Ccm system appear to function in mitochondria from at least some plants and from protists such as Reclina
monas americana (48). If any eukaryote were found to have both the Ccm system and single cysteine attachment of heme in a cytochrome c, then the present work would imply that the
Ccm system can be modified when it operates outside bacteria to cope with a single cysteine heme-binding motif.

Acknowledgments—We thank Dr. L. Thony-Meyer for the gift of plasmid pEC86, A. C. Willis for amino acid analyses, and Mark Bushell for helpful discussions.

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J. Biol. Chem. 2002, 277:33559-33563.
doi: 10.1074/jbc.M204963200 originally published online June 4, 2002

Access the most updated version of this article at doi: 10.1074/jbc.M204963200

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