Extracellular superoxide dismutase (EC-SOD) is an antioxidant enzyme that attenuates brain and lung injury from oxidative stress. A polybasic region in the carboxyl terminus distinguishes EC-SOD from other superoxide dismutases and determines EC-SOD's tissue half-life and affinity for heparin. There are two types of EC-SOD that differ based on the presence or absence of this heparin-binding region. It has recently been shown that proteolytic removal of the heparin-binding region is an intracellular event (Enghild, J. J., Thøgersen, I. B., Oury, T. D., Valnickova, Z., Hojrup, P., and Crapo, J. D. (1999) J. Biol. Chem. 274, 14818–14822). By using mammalian cell lines, we have now determined that removal of the heparin-binding region occurs after passage through the Golgi network but before being secreted into the extracellular space. Specific protease inhibitors and overexpression of intracellular proteases implicate furin as a processing protease. In vivo experiments using furin and purified EC-SOD suggest that furin proteolytically cleaves EC-SOD in the middle of the polybasic region and then requires an additional carboxypeptidase to remove the remaining lysines and arginines. A mutation in Arg12 renders EC-SOD resistant to furin processing. These results indicate that furin-dependent processing of EC-SOD is important for determining the tissue distribution and half-life of EC-SOD.

The superoxide dismutases (SOD) catalyze the dismutation of two superoxide radicals into hydrogen peroxide and oxygen. There are three mammalian superoxide dismutase (SOD) enzymes as follows: an intracellular SOD (CuZn-SOD or SOD1), a mitochondrial SOD (MnSOD or SOD2), and an extracellular SOD (EC-SOD or SOD3). EC-SOD is highly expressed in lungs and vascular tissues and is the major SOD in the extracellular space (1). In animal models, EC-SOD has been shown to attenuate tissue injury from hyperoxia (2), ischemia/reperfusion (3–6), and hemorrhage (7).

A distinguishing feature of EC-SOD is its high affinity for heparin and the extracellular matrix. Six basic amino acid residues (Arg-Lys-Lys-Arg-Arg-Arg) within the last 13 amino acid residues (8) are essential for this high affinity (9, 10). Mutations within this region lead to elevations of EC-SOD in plasma, presumably from decreased affinity toward the extracellular matrix (11–13). Removal of the polybasic region decreases the tissue half-life of EC-SOD by ~10-fold but does not affect the enzymatic activity of EC-SOD (14). Therefore, the most likely function of the polybasic region is to determine the bioavailability of EC-SOD within the extracellular matrix.

We have reported recently (15) that EC-SOD can be secreted in two forms that have a different carboxyl terminus. These different forms of EC-SOD are not because of mRNA differences but instead are due to intracellular proteolytic processing of the carboxyl terminus containing the polybasic residues. The cleaved EC-SOD has lower heparin affinity because the heparin-binding region is absent. The proteolytic removal of the carboxyl terminus could serve as a regulatory step by altering the affinity of EC-SOD for the extracellular matrix. Indeed, variability in the ratio of cleaved and intact EC-SOD has been observed among different mouse tissues (15) and during lung development (16), suggesting that there might be both spatial and temporal expression patterns of proteolytic processing of EC-SOD.

To explore fully the significance of the heparin-binding region of EC-SOD, it is necessary to determine how proteolytic processing occurs; however, neither the mechanism nor the responsible processing protease has been identified. In this current study we characterize the EC-SOD processing pathway and identify furin as a putative processing protease. The location of processing is shown to occur in a stretch of 6 consecutive basic amino acids within the last 13 amino acids and depends on the presence of an arginine residue at position 213.

**EXPERIMENTAL PROCEDURES**

Reagents—ECL Plus Western blotting detection reagents were from Amersham Biosciences. RPMI 1640 medium, the RPMI 1640 medium select amine kit, phosphate-buffered saline, Earle's balanced salt solution, and penicillin/streptomycin were from Invitrogen. Protease inhibitors of 3,4-dichloroisocoumarin, 1,10-phenanthroline (ICN), and E64 (ICN) were kept in stock solution at ~20 °C. All other reagents were purchased from Sigma. Human endothelial cells HUVEC-C (CRL-1730), RAW cells (TIB-71), transformed bronchial epithelial BEAS-2B (CRL-9609), rat glial tumor C6 (CCL-107), rat lung L2 epithelial-like cells (CCL-145), human foreskin fibroblasts (CRL-2076), CRL-1881, mouse embryoblasts (CCL-96), A549 human lung epithelial (CCL-185), human aortic smooth muscle (CRL-1999), LoVo human intestinal (CCL-229), mouse mast (CRL-8306), and RL-65 rat epithelial (CRL-10354) cells were obtained from American Type Culture Collection (Manassas, VA). Human aortic (AOSMC 5720–1) and pulmonary artery (CRL-10354) cells were obtained from American Type Culture Collection (Manassas, VA). Human aortic (AOSMC 5720–1) and pulmonary artery (CRL-10354) cells were obtained from American Type Culture Collection (Manassas, VA).
(PASMC 1018) smooth muscle cells were obtained from BioWhittaker. Human tissue was obtained from multiple autopsy specimens of apparently healthy tissue. EC-SOD was purified as described previously (17). Human recombinant EC-SOD was produced in Chinese hamster ovary cells. Mutant human furin was obtained by purifying EC-SOD from the plasma of a subject who was homozygous for the Arg213→Gly mutation. This mutation is due to a C to G transversion and is associated with markedly elevated levels of EC-SOD in the serum (18).

**Metabolic Labeling and Pulse Analysis**—Cells were grown in tissue culture plates until 80% confluent according to the ATCC or supplier guidelines. The cells were then washed twice with Earle’s balanced salt solution and incubated with 50 mM NaCl for 24 h. Cell debris was pelleted by centrifugation. Samples were cleared by adding 10 μL of immune antiserum and 40 μL of protein G-Sepharose FF (Amersham Biosciences), rotating at room temperature for 1 h, pelleting by centrifugation, and then removing the supernatant. Subsequently 10 μL of primary antiserum and 40 μL of protein G-Sepharose were added, and the mixture was rotated overnight at 4 °C. The following day the protein G-Sepharose-primary antibody complex was pelleted by centrifugation and then washed five times with 500 mM NaCl, 25 mM Tris-HCl, 5 mM EDTA, pH 7.5, with 0.5% Triton X-100, and proteinase inhibitors. Cell debris was pelleted by centrifugation. Samples were cleared by adding 10 μL of immune antiserum and 40 μL of protein G-Sepharose FF (Amersham Biosciences), rotating at room temperature for 1 h, pelleting by centrifugation, and then removing the supernatant. Subsequently 10 μL of primary antiserum and 40 μL of protein G-Sepharose were added, and the mixture was rotated overnight at 4 °C. The following day the protein G-Sepharose-primary antibody complex was pelleted by centrifugation and then washed five times with 500 mM NaCl, 25 mM Tris-HCl, 5 mM EDTA, pH 7.5, with 0.5% Triton X-100, spun through 1 μL sucrose, 500 mM NaCl, 25 mM Tris-HCl, 5 mM EDTA, pH 7.5, and finally washed twice in 10 mM Tris-HCl, 1 mM EDTA. The radiolabeled proteins were recovered by boiling in reducing SDS sample buffer with 50 mM dithiothreitol and analyzed by PAGE.

**Immunoprecipitations**—A polyclonal antiserum directed against EC-SOD and monoclonal antibody for nerve growth factor (provided by Dr. Rae Nishi, Oregon Health Sciences University) were used to recover [35S]methionine-labeled proteins as described previously (19–21). In brief, media from metabolically labeled cells were removed, and protease inhibitors were added (final concentration of 0.2 mM 3,4-dichloroisocoumarin, 0.04 mM E64, and 4 mM 1,10-phenanthroline). Cells were then washed twice with phosphate-buffered saline and scraped free in 1 mL of lysis buffer (25 mM Tris-HCl, 500 mM NaCl, 5 mM EDTA, pH 7.5, with 5% Triton X-100, and protease inhibitors). Cell debris was pelleted by centrifugation. Samples were cleared by adding 10 μL of immune antiserum and 40 μL of protein G-Sepharose FF (Amersham Biosciences), rotating at room temperature for 1 h, pelleting by centrifugation, and then removing the supernatant. Subsequently 10 μL of primary antiserum and 40 μL of protein G-Sepharose were added, and the mixture was rotated overnight at 4 °C. The following day the protein G-Sepharose-primary antibody complex was pelleted by centrifugation and then washed five times with 500 mM NaCl, 25 mM Tris-HCl, 5 mM EDTA, pH 7.5, with 0.5% Triton X-100, spun through 1 μL sucrose, 500 mM NaCl, 25 mM Tris-HCl, 5 mM EDTA, pH 7.5, and finally washed twice in 10 mM Tris-HCl, 1 mM EDTA. The radiolabeled proteins were recovered by boiling in reducing SDS sample buffer with 50 mM dithiothreitol and analyzed by PAGE.

**Protein Structure Determination**—The amino terminus was sequenced using automated Edman degradation carried out in an Applied Biosystems 477A automated protein sequencer with on-line analysis of the phenylthiohydantoin using an Applied Biosystems 120A high pressure liquid chromatography. The carboxyl terminus was determined by electrospray mass spectrometry as follows. Four μL of EC-SOD (0.9 μg/μL) was reduced to 0.5 μL of EC-SOD with 1.55 M dithiothreitol for 30 min. One μL of this solution was applied to a microseparation gel loader tip packed with Poros 1 and eluted with 5 μL of 50% MeOH and 5% formic acid in water. The sample was then analyzed on a Micro-Q-time of flight mass spectrometer with a nanoflow source using the same solvent. Carbohydrate residues were determined by measuring mass before and after treatment with the exoglycosidase neuraminidase.

**RESULTS**

**EC-SOD Processing Varies among Tissues and Cell Lines**—Comparative immunoblotting of different human organs (Fig. 1A) revealed that the total amount of EC-SOD protein was most abundant in the lung and least abundant in the brain, similar to that reported previously (22) in tissue activity assays. Moreover, immunoblotting revealed two distinct isoforms of EC-SOD migrating as 34- and 32-kDa species. We have demonstrated recently (15) that the lower molecular weight band represents EC-SOD that is missing the 13 carboxy-terminal amino acids that are essential for heparin binding. Processing of EC-SOD varied among tissues, being higher in lung, liver, and heart yet notably low in the kidney and brain. The differences persisted from experiment to experiment, suggesting that the presence of the heparin-binding region might be more important in the kidney and brain.

**To study intermediates in the processing of EC-SOD, we screened 15 mammalian cell lines (see “Experimental Procedures”) for secretion of EC-SOD. Cells were screened by pulse labeling with [35S]methionine and then immunoprecipitating the media and lysate. In addition to the rat lung L2 cell line described previously, we identified rat glial C6 cells as capable of secreting EC-SOD (Fig. 1B). Both L2 and C6 lines secreted EC-SOD with two molecular weights corresponding to the intact and proteolytically processed EC-SOD. The difference in molecular weight persisted when immunoprecipitates were treated with peptide-N-glycosidase F, which removes carbohydrate chains from N-linked residues by cleaving the aspara-
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Studies with Inhibitors of Secretory Vesicle Trafficking—Proteins that are destined for secretion pass from the endoplasmic reticulum, through the Golgi network (TGN), and then exit via the plasma membrane into the extracellular space. Although prior work (15) had suggested that EC-SOD was proteolytically processed before it reached the extracellular space, we did not know the step at which this processing occurred. To answer this question, we used several techniques in an attempt to trap proteolytically processed EC-SOD in cells. For instance, brefeldin A and nocodazole were used to block transit from the endoplasmic reticulum, and the cells were held at 20 °C to accumulate cargo proteins in the TGN. These experiments neither diminished the percentage of cleaved EC-SOD in the media nor caused an intracellular accumulation of cleaved EC-SOD (Fig. 2). Thus, proteolytic processing of EC-SOD appeared to occur after passage through the TGN. To determine whether processing occurred in a pH-sensitive vesicle, similar experiments were performed with bafilomycin; however, bafilomycin treatment did not inhibit proteolytic processing. The sensitivity of EC-SOD processing to calcium concentrations remains unknown because we were unable to remove calcium completely from the media without causing severe cell toxicity (25). Thus, proteolytic processing of EC-SOD most likely occurs after transit through the Golgi and before reaching the extracellular space.

EC-SOD Proteolytic Processing by Proprotein Convertases—Many extracellular proteins undergo proteolytic processing during their transit through the secretory pathway. To facilitate characterization and identification of the class of EC-SOD processing protease, protease inhibitors were added during the pulse-chase experiments. At multiple concentrations, neither cysteine protease inhibitors (E64), serine protease inhibitors (phenylmethylsulfonyl fluoride), nor matrix metallo-protease inhibitors (1,10-phenanthroline) were able to inhibit selectively secretion of cleaved EC-SOD without causing significant cell toxicity. Because a few extracellular proteins have been reported to be proteolytically processed by cytosolic proteasomes, the proteasome inhibitor lactacystin was added during the chase (26); however, there was no inhibition of proteolytic cleavage. The failure to prevent processing using broad inhibitors of vesicular trafficking and protease activity led us to consider more specific approaches to identify the processing protease.

Because proteolytic processing occurred near polybasic residues and after transit through the TGN, the role of the PC proteases was examined. We found that a previously described inhibitor of PC proteases, decanoyl-Arg-Val-Lys-Arg-chloromethyl ketone (Dec-RVKR-CMK), blocked proteolytic processing of EC-SOD. Furin, which is expressed at very low levels in the cell, is a PC that is highly expressed in the brain. We considered furin to trap proteolytically processed EC-SOD in cells. For instance, furin is found in the brain, and the brain cells had a similar level of processing as lung tissue (42 ± 3% versus 45 ± 3%).

Although other cell lines, such as human aortic smooth muscle cells, are known to make EC-SOD mRNA (24), we were unable to detect secretion of EC-SOD protein by immunoblotting or pulse-chase analysis in these cell lines. Because EC-SOD is a matrix protein, its secretion may depend on the presence of an intact extracellular matrix. Therefore, the cell lines were grown on Matrigel and retested for secretion of EC-SOD. Neither additional EC-SOD secretion nor a change in the ratio of intact to cleaved EC-SOD was noted when cells were grown on Matrigel. Therefore, secretion and proteolytic processing of EC-SOD appear to be unaffected by the extracellular matrix.

To study the PC family of proteases, we used multiple different approaches, all of which implicated furin as an EC-SOD processing protease. First, we examined whether a cell line that lacked PC activity would proteolytically process EC-SOD. LoVo cells, which are well known to be deficient in furin activity (27), secreted only intact EC-SOD (Fig. 4A). Second, overexpression of furin enhanced EC-SOD processing in C6 and L2 cells (Fig. 4B) and restored EC-SOD processing in the furin-deficient LoVo cells (Fig. 4C). Overexpression of a related PC protease, PC6, did not restore EC-SOD processing in LoVo.
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Fig. 4. Furin is the EC-SOD-processing protease. A, LoVo cells, which are deficient in furin activity (27), have only intact EC-SOD in both the lysate and media. B, overexpression of furin with a recombinant vaccina virus (VVfurin) increased proteolytic processing in both L2 and C6 cell lines. C, when EC-SOD is overexpressed in LoVo cells using an adenoviral vector (AdEC-SOD), there is still no processing. When LoVo cells are infected with both AdEC-SOD and VVfurin, processing increases with increasing m.o.i. of VVfurin. Co-infection with AdEC-SOD and a vaccinia viral vector that expresses the intracellular processing protease PC6 (VVPC6) did not result in EC-SOD processing.

cells. Third, we found that recombinant furin was capable of cleaving both purified and recombinant human EC-SOD in vitro (Fig. 5).

Determination of the Amino Acid Residues Involved in Proteolytic Processing of EC-SOD by Furin—Although these experiments strongly suggested that furin was involved in EC-SOD processing, several observations led us to examine further the proteolytic processing of EC-SOD by furin. First, although both recombinant furin and purified furin were capable of proteolytically processing intact EC-SOD in vitro, immunoblotting revealed that the molecular weight of the furin treated EC-SOD was higher than that of cleaved EC-SOD (Fig. 5). This suggested that furin cleaved EC-SOD after Glu209 and that there might be an additional protease that trimmed the remaining basic residues. Second, studies of EC-SOD mutants suggested that furin preferentially recognizes the cleavage site sequence Arg-Xaa-(Lys/Arg)-Arg. Based on this consensus site, one should expect furin to cleave EC-SOD after Arg210-Lys211-Lys212-Arg213. However, human pre-parathyroid hormone (32), proalbumin (33), and pro-protein C (34) also serve as furin substrates but do not have basic P4 residues, although their P3 residues are all either Arg or Lys. Thus, an alternative cleavage site for EC-SOD could be after Arg210-Lys211-Lys212-Arg213.

Re-examining the results of several studies published previously strengthens the evidence for a multistep mechanism. First, –2–4% of the population has a variant EC-SOD gene in which Arg213 is substituted to Gly213 (11–13, 35). This mutation results in the absence of cleaved EC-SOD in the serum (12, 13). Subsequently, we found that it rendered EC-SOD resistant to furin processing. Thus, Arg213 was likely to play a crucial role in the initial processing step. Second, studies of EC-SOD mutants revealed that when the carboxyl-terminal residue is arginine, the actual carboxyl-terminal amino acid in fully processed EC-SOD is Glu209, a carboxypeptidase is capable of trimming the remaining basic residues back to Glu209. Therefore, proteolytic processing of EC-SOD likely occurs via a multistep mechanism. In the first step, the endoprotease furin cleaves EC-SOD within its polybasic site at Arg213. Subsequently, a carboxypeptidase trims the remaining basic amino acid residues back to Glu209. Although the carboxypeptidase that completes these final step(s) has not yet been identified, potential candidates include carboxypeptidase D and E (28–30).

In an attempt to resolve these discrepancies, purified human recombinant EC-SOD and furin were incubated at 37 °C and then subjected to protein sequencing. Edman degradation revealed that the amino terminus was intact, suggesting that furin was indeed processing the carboxyl terminus of EC-SOD. Mass spectrometric analysis of the furin-cleaved EC-SOD revealed a number of peaks that could be related to carboxyl-terminal cleavage (Table I). Surprisingly, the mass spectrometry results suggested that the carboxyl-terminal amino acids were both Glu206-His207-Ser208-Glu209, Arg210-Lys211 and His207-Ser208-Glu209-Arg210-Lys211-Lys212. These results suggest that furin cleaves in the heparin-binding region but left some ambiguity as to which residue was crucial for furin processing. Although a silver stain of the recombinant furin preparations revealed that the predominant protein was furin, we cannot exclude that there was a small amount of carboxypeptidase activity responsible for the loss of the carboxyl-terminal arginines.

Because furin typically processes proteins with consensus sites RX(K/R)R or (K/R)XXX(K/R)R (31), we suspected that the two potential cleavage sites would be at Arg213 or Arg215. To help determine which of these two were most likely, we purified EC-SOD from the serum of a patient homozygous for a Arg213 → Gly mutation. Western blot of this protein showed predominantly unprocessed EC-SOD (Fig. 6). Furthermore, this mutation rendered the protein resistant to proteolytic processing by furin but not other proteases that can proteolytically cleave EC-SOD in vitro (e.g. trypsin). This suggested that furin processes EC-SOD at Arg213 and then a carboxypeptidase trims the remaining basic residues back to Glu209 (Fig. 7).

DISCUSSION

In this report we have identified furin as an EC-SOD processing candidate, at least for the initial step of proteolytic processing. Several lines of evidence strongly implicate furin as the processing protease in vivo. 1) LoVo cells, which are deficient in furin activity, do not secrete cleaved EC-SOD. 2) EC-SOD can be cleaved by recombinant purified furin in vitro. 3) Processing is inhibited by a specific furin inhibitor. 4) Processing occurs after passage through the Golgi, consistent with the subcellular localization of furin (31); mutation within the furin consensus sequence hinders processing both in vivo and in vitro. 5) Although the carboxyl-terminal amino acid in fully processed EC-SOD is Glu209, a carboxypeptidase is capable of trimming the remaining basic residues back to Glu209, which is the actual carboxyl-terminal amino acid in cleaved EC-SOD. Therefore, proteolytic processing of EC-SOD likely occurs via a multistep mechanism. In the first step, the endoprotease furin cleaves EC-SOD within its polybasic site at Arg213. Subsequently, a carboxypeptidase trims the remaining basic amino acid residues back to Glu209. Although the carboxypeptidase that completes these final step(s) has not yet been identified, potential candidates include carboxypeptidase D and E (28–30).
The variability of EC-SOD processing among tissues might be explained by two mechanisms. First, within organs, some cell types could secrete only intact EC-SOD and some could secrete only cleaved EC-SOD. Second, different cell types could secrete different ratios of intact to cleaved EC-SOD depending on the efficiency of furin processing within these cells. We identified three cell lines that consistently secrete different ratios of cleaved and intact EC-SOD, suggesting that individual cells might regulate intracellular proteolytic processing of EC-SOD. In LoVo cells, we were able to demonstrate that EC-SOD processing could go from 0 to 100% depending on the relative amounts of furin.

We propose that secretion of intact EC-SOD is useful for highly localizing EC-SOD activity, whereas secretion of cleaved EC-SOD is useful for a more generalized EC-SOD activity. For instance, organs that are composed of millions of small identically functioning units, such as the alveoli in the lung and the acini in the liver, may not need EC-SOD to be confined to the small functioning unit that secreted it. In these organs, we found a mix of cleaved and intact EC-SOD. On the other hand,
organs such as the brain and kidney have millions of units that do not function identically. In these organs we found mostly intact EC-SOD. The diffuse distribution of EC-SOD in the lung (36) and the highly localized distribution in the brain regions (37) have been confirmed by immunolocalization, but these studies did not distinguish between the distribution of intact and cleaved EC-SOD. Thus the putative physiologic role of EC-SOD processing remains speculative, and we are currently attempting to develop techniques that will distinguish intact from cleaved EC-SOD in situ. Using intracellular endoproteolytic cleavage to localize a protein is not unique to EC-SOD. Bone morphogenetic protein 2/4 is hypothesized to have short or long range activity, depending on the order of processing by intracellular endoprotease (38–40). Thus, we propose that tissues regulate how tightly localized their EC-SOD activity is by regulating the amount and percentage of EC-SOD that is proteolytically cleaved.

The molecular mechanisms that affect tissue distribution likely involve regulation of the polybasic residues within the intact carboxyl terminus (41–44). Two lines of evidence suggest that proteolytic processing of the heparin-binding region is paramount. First, EC-SOD variants that do not have the heparin-binding region have markedly shortened tissue half-lives (14, 45). Second, mutations within the heparin-binding region increase the plasma levels of EC-SOD (12, 46, 47). Thus, proteolytic processing of EC-SOD by furin is likely to be important in determining both the location and half-life of EC-SOD in the extracellular matrix. The clinical significance of proteolytic processing remains unknown, but it has been shown recently (13) that it inversely correlates with multiple risk factors for cardiovascular disease.

Our results demonstrate that furin is capable of proteolytic processing of EC-SOD in a two-step process. In the first step, furin cleaves EC-SOD within the heparin-binding polybasic region. A second carboxypeptidase is then necessary to remove remaining basic residues so that the final carboxyl-terminal residue is glutamic acid. Other intracellular processing proteases related to furin, such as PC7, have similar consensus sites and therefore cannot be ruled out as the authentic proc-

![Figure 6](image_url)

**FIG. 6.** The Arg213 → Gly mutation prevents furin processing. Western blotting of 10 ng of EC-SOD reveals that wild type EC-SOD exists as a mixture of cleaved and intact protein, but EDC-SOD from a subject homozygous for the Arg213 → Gly mutation is associated with predominantly unprocessed protein. Furthermore, this mutation renders EC-SOD resistant to proteolytic processing by furin but not other proteases such as trypsin.

![Figure 7](image_url)

**FIG. 7.** Suggested scheme for proteolytic processing of EC-SOD. Furin cleaves intact EC-SOD (A) within the polybasic region (cross-hatched) of the carboxyl terminus. The resulting EC-SOD protein is partially processed and has a carboxyl terminus of Arg213. B, an unknown carboxypeptidase(s) then trims the remaining carboxyl-terminal residues to glutamic acid to yield fully processed EC-SOD (C). The Arg213 → Gly mutation prevents furin from proteolytically processing EC-SOD.
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Essing proteases in vivo. The consequence of processing is that it diminishes the affinity of EC-SOD toward heparin. This potential regulatory mechanism could be exploited to change the tissue mobility of EC-SOD; however, because furin is an essential, ubiquitous enzyme, manipulation of its activity would have to be localized to specific organs.

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