Microtubule-Associated Protein MAP2 Shares a Microtubule Binding Motif with Tau Protein

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The microtubule-associated protein MAP2 is a prominent large-sized component of purified brain microtubules that, like the 36- to 38-kilodalton tau proteins, bears antigenic determinants found in association with the neurofibrillary tangles of Alzheimer's disease. The complete sequence of mouse brain MAP2 was determined from a series of overlapping cloned complementary DNAs. The sequence of the carboxyl-terminal 185 amino acids is very similar (67 percent) to a corresponding region of tau protein, and includes a series of three imperfect repeats, each 18 amino acids long and separated by 13 or 14 amino acids. A subcloned fragment spanning the first two of the 18–amino acid repeats was expressed as a polypeptide by translation in vitro. This polypeptide copurified with microtubules through two successive cycles of polymerization and depolymerization, whereas a control polypeptide derived from the amino-terminal region of MAP2 completely failed to copurify. These data imply that the carboxyl-terminal domain containing the 18–amino acid repeats constitutes the microtubule binding site in MAP2. The occurrence of these repeats in tau protein suggests that these may be a general feature of microtubule binding proteins.

MICROTUBULES ARE UBIQUITOUS in eukaryotic cells, and have a role in many cellular functions such as cell division, intracellular transport, motility, and the determination of cell shape. Analysis of microtubules prepared in vitro by successive cycles of polymerization and depolymerization shows that they consist of two major soluble proteins, the α- and β-tubulins, as well as a group of additional polypeptides known collectively as the microtubule associated proteins (MAPs). Many of these proteins promote the assembly process in vitro (*1*), and some have been identified as projections protruding from the microtubule polymer surface (*2*).

MAPs have been classified into two major groups on the basis of their size: the large molecular size MAPs (>200 kD) and the small sized tau proteins (30 to 50 kD). The large MAPs consist of several distinct polypeptides belonging to two groups. The MAP1 group contains at least three distinct members, MAP1a, MAP1b, and MAP1c (*3*), of which MAP1c has been functionally identified as cytoplasmic dynein (*4*). The MAP2 group apparently contains two members whose function in vivo is unknown, although their expression is restricted to brain (*5*) and the polypeptides are localized largely if not exclusively in dendrites (*6*).

In addition to their significance as modulators or determinants of microtubule behavior, there is evidence that antigenic determinants associated with two MAPs, that is, tau and MAP2, are contained in the neurofibrillary tangles that are characteristic of Alzheimer's disease (*7*). With a view to investigating the function of MAP2, its relation to tau protein, and its possible role in the pathology of Alzheimer's disease, we constructed a series of overlapping cloned probes encoding MAP2 from a mouse brain cDNA expression library (*5*). These clones encompassed approximately 6 kb. Here we report the complete amino acid sequence of mouse MAP2, deduced from the sequence of these and other overlapping cloned cDNAs.

The originally isolated cDNA clones contained a continuous long open reading frame, with the exception of the extreme 5′ end, in which all reading frames are closed. This does not represent a cloning artifact, since two independently isolated cDNAs encompassing this region were isolated from our unamplified cDNA libraries. To confirm that the region 5′ to the long open reading frame is indeed closed in all frames, we cloned the upstream sequences into expression vectors and analyzed the resulting fusion proteins on an SDS-polyacrylamide gel (Fig. 1). The sizes of the fusion proteins are in all cases consistent with the location of termination codons as determined from the sequence of the cloned cDNAs. We conclude that the 5′ end of our cloned cDNA corresponds to MAP2 5′ untranslated sequences. Further experiments that define the NH_2-terminus of the encoded polypeptide are described below.

Because the long open reading frame extended to the extreme 3′ end of our previously described clones, these clones did not contain sequences encoding the COOH-terminus of the MAP2 polypeptide. Therefore, a 3′-derived fragment was used to rescreen a brain cDNA library (*8*). These experiments resulted in the isolation of a further set of cDNA clones; the complete set of overlapping clones is summarized in Fig. 2, together accounting for about 7.9 kb.

The complete amino acid sequence encoded by our MAP2 cDNA clones is shown (Fig. 3). The continuity of the open reading frame contained within the overlapping

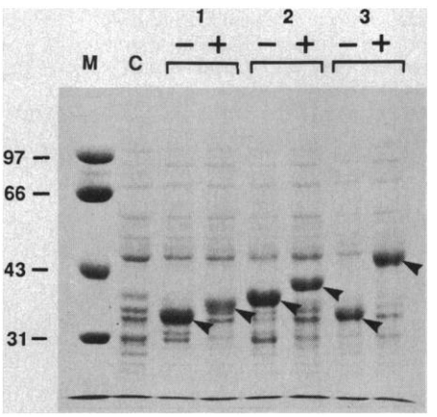

Fig. 1. The 5′ end of the MAP2 cDNA contains no long open reading frames. A DNA fragment extending from the 5′ end of clone 47 to the first internal Eco RI site (see Fig. 2) was subcloned into pATH vectors (*5*) in all three reading frames. Cultures (1 ml) of host *Escherichia coli* cells containing recombinant plasmids were induced with indole acrylic acid (10 μg/ml) and allowed to grow for 5 hours at 37°C. Cells were harvested by centrifugation, lysed directly by boiling in SDS-polyacrylamide gel loading buffer, and analyzed on an 8.5% stacking polyacrylamide gel. Lane M, molecular size markers (in kilodaltons, shown at left), and lane C, extract of untransformed host *E. coli* cells. Lanes marked 1, 2, and 3 show extracts of host *E. coli* cells bearing different pATH plasmids, each designed to express a fusion protein in one of the three reading frames. Lanes marked (−), extracts of cells bearing plasmids without cloned inserts; lanes marked (+), extracts of cells containing recombinant plasmids with inserts derived from the 5′ end of clone 47. Arrowheads indicate fusion proteins.

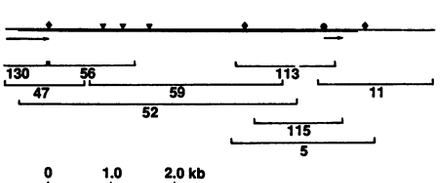

Fig. 2. Overlapping cDNA clones encoding MAP2. The long open reading frame (thick line) flanked by sequences devoid of long open reading frames is shown at the top. Arrows indicate regions encoding NH_2- and COOH-terminal sequences of the MAP2 polypeptide used in transcription and translation experiments to define the microtubule binding domain (Fig. 4). Some salient restriction sites are shown: ◆, Eco RI; ▼, Hind III; ●, Sma I.

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clones was confirmed by subcloning several regions into a series of expression vectors. In each case, a fusion protein was synthesized consistent with the predicted size of the open reading frame within the subcloned fragment; in addition, these fusion proteins were immunoreactive with a MAP2-specific antibody (*9*). Additional confirmation of the correctness of the predicted amino acid sequence derives from the fact that there is 80% sequence identity of amino acids between the sequence of mouse MAP2 reported here and a partial sequence of human MAP2 that includes amino acids 491 to 1566 (*10*). However, this human cDNA clone encodes neither the NH_2-terminus of the polypeptide nor the COOH-terminal region containing the microtubule binding site (see below).

To determine whether the first AUG codon that appears within the long open reading frame is the site of translational initiation, we cloned a restriction fragment spanning the region containing the entire 5′ closed reading frame plus the first 481 nucleotides of the long open reading frame into a vector engineered for the transcription of recombinant sequences into RNA (*11*). Capped RNA synthesized in vitro was translated in a rabbit reticulocyte cell-free system in the presence of [^{35}S]methionine (*12*), and the translation product was analyzed on an SDS-polyacrylamide gel. The data (Fig. 4, lane 2) show the presence of a 19.5-kD polypeptide, which is in reasonable agreement with translational initiation at the first AUG within the long open reading frame. Initiation at the second in-frame AUG would yield a predicted polypeptide of 14.5 kD, significantly smaller than that observed.

Comparison of the complete MAP2 sequence (Fig. 3) with other known amino acid sequences contained in the GenBank data bank did not result in the detection of significant homologies with other proteins. However, when we compared the sequence of MAP2 to that of mouse tau protein (*13*), we found a striking homology (Fig. 5). This homology encompasses the COOH-termini of the two proteins, and includes the three imperfect repeats, each of 18 amino acids and separated by 13 or 14 amino acids. The homology between MAP2 and tau is not limited to these repeats, however, as it extends over the entire 185 encoded COOH-terminal amino acids of both proteins.

The existence of a region of homology between MAP2 and tau in itself suggested that this region might contain the microtubule binding site. In addition, it has been shown that a small proteolytic fragment derived from either the NH_2- or COOH-terminus of MAP2 is the domain that binds microtubules (*14*). Therefore, we chose to express fragments from our cDNA clones encoding these regions, to see which would cycle with tubulin in microtubule assembly and disassembly experiments—the standard assay that defines microtubule-associated proteins. To do this, we subcloned two restriction fragments encoding amino acids 1 to 160 and 1621 to 1722 (Fig. 3) into vectors designed for in vitro transcription. These in vitro synthesized RNAs were each translated in a cell-free system containing [^{35}S]methionine or [^{35}S]cysteine, and the translation products were added to two-cycle purified bovine brain microtubules, which were then taken through two additional cycles of polymerization and depolymerization. At the end of each cycle, an equal portion was removed and assayed by SDS–polyacrylamide gel electrophoresis for either NH_2-terminal or COOH-terminal polypeptides that might have bound to the microtubules during the assembly process (Fig. 4). The (longer) NH_2-terminal fragment does not coassemble with the brain microtubules; however, the (shorter) COOH-terminal fragment encompassing the short homologous repeats cycles efficiently. Taken in conjunction with the observation that either the NH_2- or COOH-terminal domain of MAP2 is responsible for binding to microtubules, this experiment demonstrates that the MAP2 domain containing the 18–amino acid long repeats con-

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MADERKDEGK APHWTSASLT EAAAHPHSPE MKDQGGAGEG LSRNANGFPY REEEEGAFGE   60
HRSQGTYSDT KENGINGELT SADRETAEEV SARIVQVVTA EAVAVLKGEQ EKEAQYKDQP  120
AALPLAAEET ANLPPSPPPS PASEQTATVE EDLLTASKME FPEQEKFPSS FAEPLDKGEM  180
EFKMPSKPGE DFEHAALVPD TSKTPQDKKD LQGMEGEKLP PVPFAQTFGT NLEDRKQSTE  240
PSIVMPSIGL SAEPPAPKEP KDWFIEMPTE SKKDEWGLAA PISPGPLTPM REKDVLEDIP  300
RWEGKQFDSP MPSPFHGGSF TLPLDTMKNE RVSEGPRPFA PVFFQSDDKV SLQDPSALAT  360
SKESSKDEEP LKDKADKVAD VSISEVTTLL GNVHSPVVEG YVGENISGEV KVTTDQEKKE  420
TSAPSVQEPT LTETEPQTKL DEKSTVSIEE AVAKEEESLK LRDDKTGVIQ TSTEQSFSKE  480
DQKGQEHTID ELKQDSFPIS LEQAVTDAAM TSKTLGKVTS EPEAVSERRE IQGLFEEKTA  540
DKNKLEGAGS ATIAEVEMPF YEDKSGMSKY FETSALKEDM TRSTELGSDY YELSDSRGSA  600
QESLDTISPK NQHDEKELQA KASQPSPPAQ EAGYSTLAQS YTPGHPSELP EEPSSPQERM  660
FTIDPKVYGE KRDLHSKNKD DLTLSRSLGL GGRSAIEQRS MSINLPMSCL DSIALGFNFG  720
RGHDLSPLAS DILTNTSGSM DEGDDYLPPT TPAVEKMPCF PIESKEEEDK AEQAKVTGGQ  780
TIQVETSSES PFPAKEYYKN GTVMAPDLPE MLDLAGTRSR LASVSADAEV ARRKSVPSEA  840
MLAESSTSLP PVADESPVTV KPDSQLEDMG YCVFNKYTVP LPSPVQDSEN LSGESGSFYE  900
GTDDKVRRDL ATDLSLIEVK LAAAGRVKDE FTAEKEATPP TSADKSGLSR EFDHDRKAND  960
KLDTVLEKSE EHIDSKEHAK ESEEMGGKVE LFGLGITYDQ ASTKELITTK DTSPEKTEKG 1020
LSSVPEVAEV EPTTKADQGL DFAATKAEPS QLDIKVSDFG QMASGMNVDA GKAIELKFEV 1080
AQELTLSSEA PQEADSFMGV ESGHIKEGGK VNETEVKEKV TKPDLVHQEA VDKEESYESS 1140
GEHESLTMES LKPDEGKKET SPETSLIQDE VALKLSVEIP CPPPVSEADL STDEKGEVQM 1200
EFIQLPKEES TETPDIPAIP SDVTQPQPEA IVSEPAEVPS EEEEIEAGGE YDKLLFRSDT 1260
LQISDLLVSE SREEFVETCP GELKGVVESV VTIEDDFITV VQTTTDEGES GSHSVRFAAP 1320
AQPEEERRPR PHDEELEIEM AAEAQAEPKD GSPDAPATPE KEEVAFSEYK TETYDDYKDE 1380
TTIDDSIMDA DSLWVDTQDD DRSILTEQLE TIPKEERAEK DARRPSLEKH RKEKPFKTGR 1440
GRISTPERKV AKKEPSTVSR DEVRRKKAVY KKAELAKKSE VQAHSPSRKL ILKPAIKYTR 1500
PTHLSCVKRK TTAASGDLAQ APGAFKQAKD KVTDGISKSP EKRSSLPRPS SILPPRRGVS 1560
GDREENSFSL NSSISSARRT TRSEPIRRAG KSGTSTPTTP GSTAITPGTP PSYSSRTPGT 1620
PGTPSYPRTP GTPKSGILVP SEKKVAIIRT PPKSPATPKQ LRLINQPLPD LKNVKSKIGS 1680
TDNIKYQPKG GQVQIVTKKI DLSHVTSKCG SLKNIRHRPG GGRVKIESVK LDFKEKAQAK 1740
VGSLDNAHHV PGGGNVKIDS QKLNFREHAK ARVDHGAEII TQSPSRSSVA SPRRLSNVSS 1800
SGSINLLESP QLATLAEDVT AALAKQGL                                    1828
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Fig. 3. Deduced amino acid sequence of mouse MAP2. All cloned cDNAs contained within the region encompassed by overlapping sequences (Fig. 2) were fully sequenced on both strands by the chain terminator method of Sanger *et al.* (*23*). Nucleic acid sequence data were collated by the use of the computer programs of Staden (*24*) or our own programs, and have been transmitted to the GenBank database. The amino acid sequence predicted from the long open reading frame is shown in the one-letter code (*25*). Three imperfect 18–amino acid repeats are underlined (see Fig. 5).

stitutes the microtubule binding site. The COOH-terminal fragment used in our binding experiment encodes about 50 amino acids in addition to two of the three imperfect repeats; the relative contribution of these sequences to microtubule binding remains to be determined.

The homologous domains of tau protein and MAP2 are both rich in basic amino acids, with net charges of 12 and 18, respectively, within the COOH-terminal 185 amino acids. Current evidence suggests that MAP2 binds to the COOH-terminal domains of both α- and β-tubulin (*15*). In all known mammalian α- and β-tubulin isotypes the COOH-terminal regions are highly acidic (*16*). These data therefore provide an explanation for the observation that (i) the ionic interaction between tubulin and MAP2 and tau is disrupted by high concentrations of salt (*17*), and (ii) a basic peptide of about 28 kD derived from proteolytic cleavage of MAP2 specifically interacts with microtubules in vitro (*14*).

Sequence analysis of cloned cDNAs encoding tau proteins has shown that the size heterogeneity of these polypeptides is due at least in part to alternative splicing that results in some molecules containing extended COOH-terminal sequences (*13*). In the case of mouse MAP2, restriction mapping of 12 independently isolated overlapping cDNA clones encompassing the region containing the translational termination codon failed to detect the presence of any large-scale sequence heterogeneity in this region. However, there is tentative evidence for differential RNA splicing of MAP2 transcripts in rat brain, which results in the expression of a 70-kD polypeptide related to the NH_2-terminal portion of MAP2 (*18*). In contrast, the closely related large forms of MAP2 may well be distinguished by differences in phosphorylation. The MAP2 sequence (Fig. 3) contains many potential phosphorylation sites, but no identifiable sites exist in close proximity to the microtubule binding domain.

The predicted molecular size of MAP2 is 198,978 daltons, which is similar to the value based on equilibrium sedimentation and gel filtration experiments, but considerably lower than the apparent size based on migration in SDS-polyacrylamide gels. Circular dichroism measurements and the nuclear magnetic resonance spectrum of MAP2 indicate that the protein is unordered and extended in structure with little in the way of α helix, coils, or β sheets (*19*). The absence of ordered structure in the MAP2 polypeptide probably accounts for its thermal stability and is consistent with the lack of secondary structure predicted on the basis of analysis of our sequence data obtained with the programs of Garnier *et al.* (*20*) and Chou and Fasman (*21*). The only significant structural features revealed by this analysis lie in the COOH-terminal microtubule binding domain. In this region, all three of the 18–amino acid repeats fall on predicted reverse turns in the protein. A similar secondary structure is predicted for the corresponding region in tau protein.

Because an in vitro synthesized polypeptide containing two of the three 18–amino acid repeats copurifies efficiently with microtubules through at least two cycles of polymerization and depolymerization, it seems possible that a single unit of the 18–amino acid repeat might be sufficient to bind to a tubulin molecule. In that event, the repeated units may serve to cross-link several tubulin molecules within the microtubule polymer. The size and spacing of the repeats are consistent with this hypothesis, given an interpeptide bond distance of 0.38 nm and a 4-nm spacing of tubulin subunits in the microtubule polymer. Such an interaction could explain the ability of MAP2 and tau to promote microtubule assembly in vitro, and suggests that, like tau protein (*22*), at least one function of MAP2 is to enhance microtubule stability and nucleation in vivo.

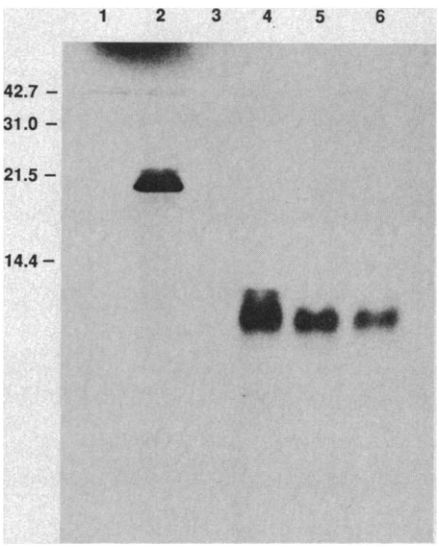

Fig. 4. The microtubule binding site of MAP2 is the COOH-terminal domain including the 18–amino acid repeats. Two fragments, one extending from the 5′ end of clone 47 to the proximal Eco RI site and a second extending from the Sma I site contained in clone 115 to the 3′ end of this clone (see Fig. 2) were subcloned into pGEM vectors (*11*) and used as templates for the preparation of capped mRNA (*26*) transcribed in vitro from the prokaryotic SP6 promoter contained in these vectors. An initiator methionine codon was supplied to the fragment derived from clone 115 via the AUG contained in the polylinker of the pGEM vector. The capped, in vitro synthesized mRNAs were used as templates for in vitro translation reactions with either micrococcal nuclease–treated rabbit reticulocyte lysate (*12*) or wheat germ embryo–derived (*27*) cell-free systems containing either [^{35}S]methionine or [^{35}S]cysteine. After translation in vitro, the translation reactions were mixed and centrifuged at 200,000*g* (Beckman TL-100). The supernatant was added to a preparation of two-cycle purified, depolymerized bovine brain microtubules (*28*), which was then subjected to two further cycles of polymerization and depolymerization. At the end of each depolymerization reaction, a portion (containing 1/20 of the total mixture) was removed for analysis on a 14% stacking SDS-polyacrylamide gel. Lanes 1 and 3, control translation reactions (no added RNA). Lane 2, translation of in vitro synthesized RNA corresponding to the 160 NH_2-terminal amino acids of MAP2. Lane 4, translation of in vitro synthesized RNA corresponding to amino acids 1621 to 1722. Lanes 5 and 6, analysis of microtubules after one (lane 5) and two (lane 6) successive cycles of polymerization and depolymerization in the presence of equimolar quantities of in vitro translated NH_2-terminal and COOH-terminal peptides. The (larger) NH_2-terminal peptide is not present in cycled microtubule preparations. Molecular markers (in kilodaltons) are shown at the left.

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1608        1618        1628        1638        1648        1658
   GTPPSYSSRT PGTPGTPSYP RTPGTPKSGI LVPSEKKVAI IRTPPKSPAT PKQLRLINQP
                 ** ****                        ****   ********     *     *
   KSGERSGYSS PGSPGTPGSR SRTPSLPTPP TREP-KKVAV VRTPPKSPAS KSRLQTAPVP
 121         131         141         151         160         S 171
1668        1678        1688        1698        1708        1718
   LPDLKNVKSK IGSTDNIKYQ PKGGQVQIVT KKIDLSHVTS KCGSLKNIRH RPGGGRVKIE
    ****** ** **** * * * * ** ****  *  *** *** ***** ** *  **** *
   MPDLKNVRSK IGSTENLKHQ PGGGKVQIVY KPVDLSKVTS KCGSLGNIHH KPGGGQVEVK
 181         191         201         211         221         231
1728        1738        1748        1758        1768        1778
   SVKLDFKEKA QAKVGSLDNA HHVPGGGNVK IDSQKLNFRE HAKARVDHGA EIITQSPSRS
   * *****    * * *****   ******* * **  ** ***  ***  **** **   **  *
   SEKLDFKDRV QSKIGSLDNI THVPGGGNKK IDTHKLTFRE NAKAKTDHGA EIVYKSPVVS
 241         251         261         271         281         291
1788        1798        1808        1818        1828
   SVASPRRLSN VSSSGSINLL ESPQLATLAE DVTAALAKQG L
      *** *** *** ***     ********   * * ***** *
   GDTSPRHLSN VSSTGSIDMV DSPQLATLAD EVSASLAKQG L
 301         311         321         331         341
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Fig. 5. Homology between COOH-terminal sequences encoding MAP2 and tau protein: the COOH-terminal 220 amino acids are compared (*25*). Asterisks denote homology; the three 18–amino acid repeats are underlined. A single amino acid "deletion" and a single amino acid "insertion" have been introduced into the tau sequence to reveal two additional short regions of homology (encompassing residues 1618 to 1624 and residues 1643 to 1656 of the MAP2 sequence).

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Ovothiol Replaces Glutathione Peroxidase as a Hydrogen Peroxide Scavenger in Sea Urchin Eggs

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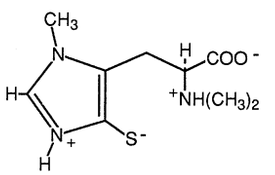
Despite its potential toxicity, H_2O_2 is used as an extracellular oxidant by *Strongylocentrotus purpuratus* eggs to cross-link their fertilization envelopes. These eggs contain 5 m*M* 1-methyl-N^{α},N^{α}-dimethyl-4-mercaptohistidine (ovothiol C), which reacts with H_2O_2. In consuming H_2O_2 and being reduced by glutathione, ovothiol acts as a glutathione peroxidase and replaces the function of the enzyme in eggs. The ovothiol system is more effective than egg catalase in destroying H_2O_2 at concentrations produced during fertilization and constitutes a principal mechanism for preventing oxidative damage at fertilization.

THE USEFUL BUT POTENTIALLY DESTRUCTIVE nature of oxygen in biological systems is especially well illustrated in sea urchin fertilization. Within minutes of gamete membrane fusion, sea urchin eggs form a protective fertilization envelope that is cross-linked by dityrosyl residues (*1–3*). Dityrosine formation, catalyzed by a secreted ovoperoxidase (*4*), requires H_2O_2 as an extracellular oxidant; H_2O_2 is produced in a respiratory burst that lasts during early embryonic activation (*5*). Thus the embryo creates a potential hazard by using a powerful oxidant like H_2O_2 at the beginning of its development. Sea urchin eggs and embryos contain one of the family of 4-mercaptohistidines (*6–9*), biological aromatic thiols with novel redox properties (*7, 10–13*). Ovothiols were discovered as cofactors for a cyanide-resistant reduced pyridine nucleotide [NAD(P)H] oxidase of ovoperoxidase (*11*), although this does not appear to be their physiological role (*7*). We show that the 5 m*M* ovothiol C (OSH) present in sea urchin eggs and embryos may function in the control of H_2O_2 toxicity by providing a nonenzymatic glutathione peroxidase (GPx)–like activity to protect the early embryo.



Ovothiol C

OSH is rapidly oxidized to ovothiol disulfide (OSSO) by H_2O_2, suggesting that OSH may control H_2O_2 toxicity at fertilization. Since glutathione is present in the reduced state (GSH) in eggs (*14*) and since the midpoint redox potential of ovothiol is 84 mV positive with respect to glutathione (*12*), ovothiol will be present as OSH in vivo. Oxidized ovothiol and oxidized glutathione (GSSG) are formed in the presence of H_2O_2 (*6, 12*)

$$2\ OSH + H_2O_2 \rightarrow OSSO + 2\ H_2O \quad (1)$$

$$2\ GSH + H_2O_2 \rightarrow GSSG + 2\ H_2O \quad (2)$$

The rate of formation of each oxidized species was first-order in H_2O_2 and thiol, yielding second-order kinetic constants (20 m*M* tris-Hepes, *p*H 7.2, 30°C) of 2.0 $s^{-1}\ M^{-1}$ (reaction 1) or 0.43 $s^{-1}\ M^{-1}$ (reaction 2). Whereas an increase in *p*H from 7.2 to 7.6 doubled the rate of oxidation of GSH by H_2O_2, the same *p*H change increased the rate of reaction of OSH with H_2O_2 by less than 10%; this is at least in part due to the difference in thiolate pK_a for GSH (8.6) (*15*) and OSH (2.3) (*10*). The rates of reaction of OSH and GSH with H_2O_2 were not dependent on the presence of divalent

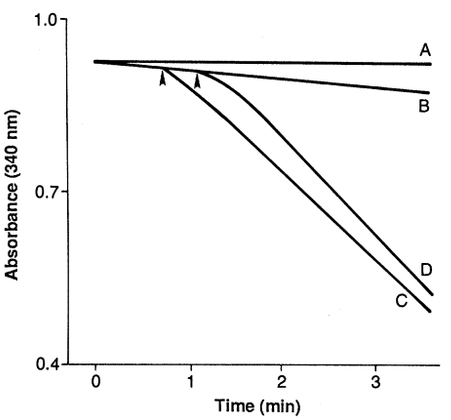


Fig. 1. Glutathione peroxidase activity of ovothiol. GPx was measured by the disappearance of NADPH (*26*) (Table 1). Reactions were initiated with H_2O_2; OSH or glutathione peroxidase (Sigma) were added later, as shown. Line A, background consumption of NADPH; line B, control, with 0.2 m*M* GSH present at time 0; line C, addition of 1 m*M* OSH at arrowhead; and line D, addition of 1 unit of bovine erythrocyte GPx (Sigma) at arrowhead.

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