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Our research during the project period was directed towards three fundamental aspects of PCs: (i) metal-chelation by PCs, (II) sulfide complexes of PCs and (III) biosynthesis of PCs. We have made thorough investigations of the metal-binding properties of PCs and published data that provides benchmark information on the Ag(I), Cu(I), Cd(II), Hg(II) and Pb(II)-binding characteristics of these plant metal binding peptides We have carried out detailed investigations on the sulfide complexes of PCs (PCs) that profoundly influence the metal-binding capacity of these peptides. In addition, these complexes have the potential to be very useful reagents for the detoxification of a variety of organic contaminants. Using a complementation cloning strategy, we have isolated a gene that is required for Cd tolerance and PC production. Although this gene did not turn out to be PC synthase, it provides us a good pictur of the role of heme biosynthesis in PC production.

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FINAL REPORT

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MOLECULAR MECHANISMS OF BIOSYNTHESIS OF METAL-BINDING PCs

Principal Investigator: Rajesh K. Mehra, University of California, Riverside, CA 92521

Grant No. : F49620-94-1-0047 P00001

Summary of the Report

Our research during the project period was directed towards three fundamental aspects of PCs: (i) metal-chelation by PCs, (ii) sulfide complexes of PCs and (iii) biosynthesis of PCs. We have made thorough investigations of the metal-binding properties of PCs and published data that provides benchmark information on the Ag(I), Cu(I), Cd(II), Hg(II) and Pb(II)-binding characteristics of these plant metal binding peptides. We have carried out detailed investigations on the sulfide complexes of PCs (PCs) that profoundly influence the metal-binding capacity of these peptides. In addition, these complexes have the potential to be very useful reagents for the detoxification of a variety of organic contaminants. Using a complementation cloning strategy, we have isolated a gene that is required for Cd tolerance and PC production. Although this gene did not turn out to be PC synthase, it provides us a good picture of the role of heme biosynthesis in PC production.

Metal-binding characteristics of PCs

The ability of PCs to detoxify heavy metals depends on their metal-chelation properties. Although several studies have shown that a variety of metals can induce the synthesis of PCs, relatively little was known about the metal-binding stoichiometries and affinities of these peptides. We undertook a systematic approach to study the metal-binding properties of PCs using a variety of techniques. For most of these studies, three PCs differing in chain-length were used. The PCs with the structures $(\gamma\text{-Glu-Cys})_2\text{Gly}$, $(\gamma\text{-Glu-Cys})_3\text{Gly}$ and $(\gamma\text{-Glu-Cys})_4\text{Gly}$ were used for these experiments. Summaries of studies on individual metals are presented below.

Ag(I)-binding studies

Ag(I)-binding characteristics of PCs were determined by (i) UV/VIS spectrophotometry, (ii) luminescence spectroscopy at 77°K and by (iii) reverse-phase HPLC. The three techniques yielded similar results. apoPCs exhibit featureless absorption in the 220-340 nm range. The binding of Ag(I) to PCs induced the appearance of specific absorption shoulders. The titration end point was indicated by the flattening of the characteristic absorption shoulders. Similarly, luminescence at 77°K due to Ag(I)-thiolate clusters increased with the addition of graded Ag(I) equivalents. The luminescence declined when Ag(I) equivalents in excess of the saturating amounts were added to the peptides. At neutral pH, $(\gamma\text{-Glu-Cys})_2$Gly, $(\gamma\text{-Glu-Cys})_3$Gly and $(\gamma\text{-Glu-Cys})_4$Gly bind 1.0, 1.5 and 4.0 equivalents of Ag(I), respectively. The Ag(I)-binding capacity of $(\gamma\text{-Glu-Cys})_2$Gly and $(\gamma\text{-Glu-Cys})_3$Gly was increased at pH 5.0 and below so that Ag(I)/-SH ratio approached 1.0. A similar pH-dependent binding of Ag(I) to glutathione was also observed. The increased Ag(I)-binding to PCs at lower pH may be of physiological significance as these peptides accumulate in acidic vacuoles. We have also collected lifetime data on Ag(I)-PCs. The relatively long decay-times (~0.1-0.3 msec) accompanied with a large Stokes shift in the emission band are indicative of spin-forbidden phosphorescence.

Cu(I)-binding studies

Cu(I)-binding characteristics of PCs were studied by absorption and emission spectroscopy. In addition, room temperature luminescence attributable to Cu(I)-thiolate clusters was used to probe the transfer of Cu(I) from Cu(I)-glutathione complex to rabbit liver thionein-II

and PCs. Reconstitutions were also performed using CuCl. The Cu(I)-binding stoichiometries of metallothionein or PCs were generally independent of the Cu(I) donor. However, the luminescence of the reconstituted metallothionein or PCs was higher when Cu(I)-GSH was the donor. This higher luminescence was presumably due to the stabilizing effect of GSH on Cu(I)-thiolate clusters. As expected, 12 Cu(I) ions were bound per molecule of metallothionein. The Cu(I)-binding to PCs depended on their chain length; the binding stoichiometries being 1.25, 2.0 and 2.5 for $(\gamma Glu\text{-}Cys)_2Gly$, $(\gamma Glu\text{-}Cys)_3Gly$ and $(\gamma Glu\text{-}Cys)_4Gly$ respectively at neutral pH. A reduced stoichiometry for the longer PCs was observed at alkaline pH. No GSH was found to associate with PCs by a gel filtration assay. The Cu(I)-binding to $(\gamma Glu\text{-}Cys)_2Gly$ and $(\gamma Glu\text{-}Cys)_3Gly$ occurred in a biphasic manner in the sense that the relative luminescence increased approximately linearly with the amount of Cu(I) up to a certain molar ratio whereafter luminescence increased dramatically upon the binding of additional Cu(I). The luminescence intensity declined once the metal binding sites were saturated. In analogy with the studies on metallothioneins, biphasic luminescence suggested the formation of two types of Cu(I) clusters in PCs.

Hg(II)-binding studies

Optical spectroscopy and reverse-phase high-performance liquid chromatography (HPLC) were used to investigate the binding of Hg(II) to PCs. Glutathione-mediated transfer of Hg(II) into PCs and the transfer of the metal ion from one PC to another was also studied using reverse-phase HPLC. The saturation of Hg(II)-induced bands in the UV/visible and circular dichroism spectra of $(\gamma Glu\text{-}Cys)_2Gly$ suggested the formation of a single Hg(II)-binding species of this

peptide with a stoichiometry of one metal ion per peptide molecule. The separation of apo-(γGlu-Cys)$_2$Gly from its Hg(II)-derivative on a C_{18} reverse-phase column also indicated the same metal-binding stoichiometry. The UV/visible spectra of both (γGlu-Cys)$_3$Gly and (γGlu-Cys)$_4$Gly at pH 7.4 showed distinct shoulders in the ligand-to-metal charge-transfer region at 280-290 nm. Two distinct Hg(II)-binding species, occurring at metal-binding stoichiometries of around 1.25 and 2.0 Hg(II) ions per peptide molecule, were observed for (γGlu-Cys)$_3$Gly. These species exhibited specific spectral features in the charge-transfer region and were separable by HPLC. Similarly, two main Hg(II)-binding species of (γGlu-Cys)$_4$Gly were observed by UV/visible and circular dichroism spectroscopy at metal-binding stoichiometries of around 1.25 and 2.5, respectively. Only a single peak of Hg(II)-n_4 complexes was resolved under the conditions used for HPLC. The overall Hg(II)-binding stoichiometries of PCs were similar at pH 2.0 and at pH 7.4 indicating that pH did not influence the final Hg(II)-binding capacity of these peptides. The reverse-phase HPLC assays indicated a rapid transfer of Hg(II) from glutathione to PCs. These assays also demonstrated a facile transfer of the metal ion from shorter to longer chain PCs. The strength of Hg(II) binding to glutathione and PCs followed the order: γGlu-Cys-Gly < (γGlu-Cys)$_2$Gly < (γGlu-Cys)$_3$Gly < (γGlu-Cys)$_4$Gly.

Pb(II)-binding studies

UV/visible and circular dichroism (CD) spectroscopy proved useful for studying the binding of Pb(II) to PCs. Saturation of the Pb(II)-induced charge-transfer bands indicated that both (γGlu-Cys)$_2$Gly and (γGlu-Cys)$_3$Gly bound one metal ion per peptide molecule. However, (γGlu-Cys)$_4$Gly formed two distinct species with stoichiometries of one and two Pb(II) ions per

peptide molecule, respectively. The optical spectra of $Pb(II)_1$-(γGlu-Cys)$_4$Gly were similar to those of $Pb(II)_1$-(γGlu-Cys)$_3$Gly, whereas the spectra of $Pb(II)_2$-(γGlu-Cys)$_4$Gly were similar to those of $Pb(II)_1$-(γGlu-Cys)$_2$Gly. Since cysteinyl thiolates are the likely ligands for Pb(II) in PCs, Pb(II) appears to form two-, three- and four-coordinate complexes with PCs depending on their chain length. Furthermore, Pb(II) may exhibit multiple coordination in longer chain PCs as indicated by the formation of two Pb(II)-binding species of (γGlu-Cys)$_4$Gly. The transfer of Pb(II) from glutathione to PCs and from shorter chain to longer chain PCs was also demonstrated.

Metal-binding characteristics of PC-analogs

The present studies were undertaken to explore the possibility of using synthetically designed genes encoding PC analog (Glu-Cys)$_n$Gly peptides in transgenic plants for phytoremediation. As a first step, we studied the metal-chelating characteristics of a synthetically prepared PC analog peptide (Glu-Cys)$_2$Gly to determine if a gene encoding such a peptide might be useful in phytoremediation. Studies with Cd(II), Hg(II) and Pb(II) show that the synthetic (Glu-Cys)$_2$Gly peptide exhibits metal-chelating properties similar to the PC (γGlu-Cys)$_2$Gly. GSH-bound metals were also shown to be quantitatively transferred to (Glu-Cys)$_2$Gly. The Cd(II)-form of the synthetic (Glu-Cys)$_2$Gly peptide like PCs was able to form stable complexes with sulfide. The spectroscopic properties of (Glu-Cys)$_2$Gly-coated complexes of CdS were comparable to those exhibited by (γGlu-Cys)$_2$Gly-coated CdS particles. Both (γGlu-Cys)$_2$Gly and (Glu-Cys)$_2$Gly exhibited a Cd-binding stoichiometry of 0.5 Cd per peptide molecule. UV/VIS, HPLC and mass-

spectral analyses indicated that one Hg(II) ion was chelated by each molecule of $(\gamma Glu\text{-}Cys)_2Gly$ or $(Glu\text{-}Cys)_2Gly$. Each molecule of $(\gamma Glu\text{-}Cys)_2Gly$ or $(Glu\text{-}Cys)_2Gly$ bound one atom of Pb(II).

Sulfide-complexes of PCs and related molecules

There have been a few publications claiming that PCs may have a limited role in Cd tolerance. The metal resistance levels may depend on factors such as the PC concentrations, their chain length and ability to incorporate labile sulfide. We have isolated and characterized a highly Cd(II)-resistant mutant of *C. glabrata* to address these questions. This mutant exhibited lower GSH and PC concentrations than the wild-type strain at similar Cd(II) exposure. However, spectroscopic, chemical and direct microscopic analyses demonstrated extremely high levels of CdS quantum crystallites in the resistant strain. The CdS crystallites were formed in the cytosol but finally accumulated in the vacuoles. Cd(II)-stimulated sulfide production required sulfate and was inhibited by both cysteine and methionine. Inhibition of GSH synthesis sensitized the resistant strain to Cd(II) indicating that GSH still provided primary defense against the metal ion.

As explained below, detailed studies of the sulfide complexes were carried out to determine the influence of sulfide on metal-binding characteristics.

Nature of GSH and PC-capped CdS complexes

The incorporation of inorganic sulfide into cadmium-glutathione (GSH) led to the formation of a variety of GSH-capped CdS complexes that differed in sulfide/Cd(II) ratios, optical spectroscopic properties and Cd(II)-binding capacity of GSH. The size fractionation of GSH-CdS complexes indicated that the Cd(II)/GSH molar ratio increased from a minimum of 0.3 to a maximum of 25 as

sulfide/Cd(II) molar ratio increased from 0 to ~1.0 equivalent. The absorption shoulders in the 290-400 nm range, photoluminescence in the 400-550 nm range and the ability to reduce methylviologen indicated that these GSH-CdS complexes behaved like semiconductor nanocrystallites. The predicted radii of GSH-capped CdS nanocrystallites varied from 10.8 to 17.3 Å. Unlike GSH, PCs formed CdS crystallites that appeared uniform in size as was indicated by their similar optical properties. Although the properties of PC-capped CdS complexes were controllable by altering the amounts of sulfide titrated, sulfide-induced transitions in the electronic absorption spectrum (indicative of the crystallite size) were limited to the blue of 318 nm. Thus, the maximum predicted radius of PC-capped crystallites was 11.8 Å. The titration of PCs into GSH-capped CdS crystallites led to the replacement of GSH with PCs. Interestingly, the displacement of GSH by PCs did not alter the size of CdS particles as indicated by lack of changes in emission λ_{max} or in the characteristic absorption shoulder at 358 nm. However, emission yields were quantitatively decreased upon displacement of GSH with PCs.

Cysteine alone mediates synthesis of CdS particles

The synthesis of CdS bionanocrystallites has been studied by reacting Cd(II)-cysteine complexes with inorganic sulfide. Cysteine-mediated synthesis of CdS bionanocrystallites proceeded slightly faster at 45°C than at room temperature. The sizes of these CdS bionanocrystallites, as determined by optical spectroscopy, increased with increasing initial sulfide/Cd(II) ratios. Analyses of size-distribution showed significant heterogeneity in sizes only at sulfide/Cd(II) ratios of 0.25. An ethanol precipitation procedure was developed to remove unreacted Cd(II)-cysteine complexes. This procedure also resulted in the isolation of CdS bionanocrystallites (bioNCs) that appeared uniform in size and chemical composition. Cysteine

behaved like glutathione insofar as the size-range of CdS particles was concerned. However, cysteine-mediated synthesis of CdS bioNCs resulted in uniformly sized-particles as has been observed previously with PCs. Cysteine-capped CdS particles exhibited pH-dependent changes in their properties. pH-induced changes were more pronounced in emission than in absorption spectra. Photocatalytic activities of these bioNCs were indicated by reduction of three dyes. Irradiation of methylviologen, basic fuchsin and naphthol blue black at 366 nm in the presence of cysteine-capped CdS bioNCs caused reduction of these dyes. Samples exhibiting higher luminescence were also more active in the photocatalysis experiments.

GSH and cysteine also mediate synthesis of ZnS nanoparticles

The titration of increasing equivalents of inorganic sulfide into preformed Zn-glutathione led to the appearance of UV/VIS spectral features attributable to ZnS nanocrystallites. Glutathione-ZnS complexes upon irradiation caused reduction of methylviologen confirming their semiconductor properties. Size-fractionation of glutathione-ZnS samples on a gel filtration column showed the formation of a range of complexes whose spectral properties were correlated with the sulfide content. The stoichiometry of Zn to glutathione increased from 1:2 to a maximum of about 7:1 as the titrated sulfide increased from 0 to 2.0 molar equivalents indicating a substantial increase in the Zn-binding capacity of glutathione upon incorporation of sulfide. Spectral characteristics of GSH-capped ZnS nanocrystallites were significantly influenced by pH and by the stoichiometry of Zn, sulfide and glutathione in the complex. The samples containing least glutathione and highest sulfide showed maximal luminescence at pH 6, whereas those with higher glutathione and lower sulfide content showed maximal luminescence at pH 11.

Cysteine-capped ZnS bionanocrystallites (bioNCs) were prepared by titrating sodium sulfide into preformed Zn-cysteine complexes. Only a maximum of ~40 % of Zn(II) in Zn-cysteine complex was converted into ZnS bioNCs when the reaction was carried out at room temperature for 30 min. However, incubation of the reaction mixture at 45°C for 60 min significantly enhanced the production of ZnS bioNCs as a maximum of ~75% of Zn(II) was converted into bioNCs. Cysteine capping produced NCs that were smaller than those capped by glutathione. Furthermore, cysteine-capped ZnS exhibited a narrow range of size distribution as determined by UV/VIS spectroscopy of the bioNCs separated on a size-fractionation column. Unreacted Zn-cysteine complex could be removed from ZnS bioNC preparations by selective precipitation with ethanol. The precipitation procedure also led to the isolation of ZnS bioNCs that appeared more uniform by gel-filtration analysis. Ethanol precipitation procedures allowed preparation of large quantities of powdered bioNCs which retained their colloidal nature upon resuspension in water or buffers. pH titration experiments indicate that the average size of the particles was smallest in the pH range 7-10, but the size increased as the samples were made acidic or alkaline. Cysteine-capped bioNCs were capable of causing photoreduction of methylviologen, basic fuchsin and naphthol blue black.

Biosynthesis of PCs

In vitro assays for PC synthase have generally been less reliable making it difficult to purify the enzyme. We took a genetic approach to identify PC synthase. A *Candida glabrata* cadmium-sensitive mutant partially defective in glutathione production and exhibiting a complete absence of PCs was used to clone a gene required for Cd tolerance. Transformation of the Cd-sensitive mutant with a genomic library from the wild-type *C. glabrata* led to the cloning of a

gene that restored Cd tolerance and formation of Cd-glutathione and Cd-PC complexes. The cloned gene showed high levels of nucleic acid and protein sequence homology to the *HEM2* genes, encoding porphobilinogen synthases, from several sources. It was shown that the *C. glabrata* Cd-sensitive mutant indeed exhibited a significant reduction in porphobilinogen synthase levels. The cloned *C. glabrata* gene complemented a *hem2* mutant of *Saccharomyces cerevisiae* and restored porphobilinogen synthase activity in the mutant. The Cd sensitive mutant predictably showed decreased levels of sulfite reductase that requires siroheme, a metabolite produced in the heme biosynthetic pathway. The addition of cysteine, but not methionine, increased glutathione levels and Cd tolerance of both the wild-type and the mutant strain. However, addition of hemin chloride and methionine together restored Cd tolerance indicating that heme was required for transsulfuration of homocysteine to cysteine.

Personnel supported

The funds provided by AFOSR were used to provide partial salary supports to graduate students, a postdoctoral scientist and an undergraduate researcher.

Publications (supported in part by AFOSR)

(1993-1997)

1. Mehra, R.K., Mulchandani, P. and Hunter, T.C. (1994) Role of CdS quantum crystallites in cadmium resistance in *Candida glabrata*. **Biochem. Biophys. Res. Comm.** 200, 1193-1200

2. Mehra, R.K. and Mulchandani, P. (1995) Glutathione-mediated transfer of Cu(I) into PCs. **Biochem. J.** 307, 697-705

3. Mehra, R. K., V.R. Kodati and Abdullah, R. (1995) Chain-length dependent Pb(II)-coordination in PCs. **Biochem. Biophys. Res. Comm.** 215, 730-736

4. Mehra, R.K., Tran, K., Scott, G.W., Mulchandani, P. and Saini, S.S. (1996) Ag(I)-binding to PCs. **J. Inorg. Biochem.** 61, 125-142.

5. Mehra, R.K., Miclat, J., Kodati, R.V., Abdullah, R., Hunter, T.C. and Mulchandani, P. (1996) Optical spectroscopic and reverse-phase HPLC analyses of Hg(II)-binding to PCs. **Biochem. J.** 314, 73-82

6. Tripathi, R.D., Yunus, M. and Mehra, R.K. (1996) PCs and phytometallothioneins: The

potential of these unique metal detoxifying systems in plants.

Physiol. Mol. Biol. Plants. 2, 101-104

7. Bae, W., Abdullah, R. Henderson, D. and Mehra, R.K. (1997) Characteristics of glutathione-capped ZnS nanocrystallites. **Biochem. Biophys. Res. Comm.** 237, 16-23

8. Bae, W. and Mehra, R.K. (1997) Metal-binding characteristics of a PC analog (Glu-Cys)$_2$Gly. **J. Inorg. Biochem.** 68, 201-210

9. Bae, W. and Mehra, R.K. (1998) Properties of glutathione- and PC-capped CdS bionanocrystallites. **J. Inorg. Biochem. (In press).**

10. Hunter, T. C. and Mehra, R.K. (1998) A role for *HEM2* in Cd tolerance. **J. Inorg. Biochem. (In press).**

11. Bae, W. and Mehra, R.K. (1997) Preparation and characterization of uniformly-sized ZnS bionanocrystallites capped with cysteine (submitted).

12. Bae, W. , Abdullah, R. and Mehra, R.K. (1997) Cysteine-mediated synthesis of CdS Bionanocrystallites (submitted).

13. Mehra, R.K. (1997) Biosynthesis and metal-binding characteristics of PCs. "Pollution

stress: indication, mitigation and conservation" Kluwer Academics, Editors: Yunus *et al* (submitted).

Interactions/Transitions

Meetings/Conferences

- American Society of Biochemistry and Molecular Biology, San Diego, California, May 30-June 3, 1993. "Mechanisms of Cd resistance in *Candida glabrata*."

- Mid-Atlantic yeast meeting, May 25-27, 1994. "Cd(II)-stimulated biosynthesis of CdS crystallites and their vacuolar targeting in yeast."

- Experimental Biology, April 25, 1994. "Mechanism of cadmium resistance in Candida glabrata."

- Gordon Conference 1995. "Glutathione-mediated transfer of Cu(I) into phytochelatins."

- UC Irvine 1995. "Biochemistry and genetics of metal detoxification."

- Genetics Society of America 1994. "Role of the vacuole in metal tolerance."

- Gordon Research Conference, January 25-29, 1996, Ventura, California, "Metal-binding properties of phytochelatins."

- Society of Toxicology, March 14, 1996, Anaheim, California, "Metal-binding properties of phytochelatins (PCs)."

- UC Toxic Substance Research and Teaching Program, March 29-30, 1996, UC Santa Cruz, "Phytoremediation of toxic metals using designer genes."

- International Conference on Environmental Pollution & Plants, Lucknow, India, November 26-30, 1996, "Biosynthesis and metal-binding properties of Phytochelatins."

- Guru Nanak Dev University, India, December 2, 1996, " □-aminolevulinic acid dehydratase: An enzyme with two faces?"

- Panjab University, India, December 3, 1996, "Biophysics and molecular biology of metal tolerance."

- Simbhawli College, India, December 6, 1996, "Metal tolerance in plants."

- 2nd Symposium on Gene Expression, March 18, 1997, "A role for *HEM2* in Cd

tolerance."

- UC Toxic Substances Research & Teaching Program, April 11-12, 1997, "Construction of synthetic genes for phytoremediation of toxic metals"

- Environmental Chemistry Seminar Series, Oct. 7, 1997 "Of yeasts, semiconductors and bioremediation."

- American Society of Biochemistry and Molecular Biology Fall Symposium, Lake Tahoe, Oct. 24-27, 1997, "GSH- and phytochelatin-capped CdS bionanocrystallites: Preparation and characterization."

- NC Workshop: Semiconductor nanocrystals and nanocrystal-based composites, Los Alamos National Laboratory, Nov. 23, 1997, "Bio-nanocrystals: Synthesis, optics and bio-applications."

- International Union of Biochemistry and Molecular Biology, San Diego, August 28, 1997, "Metal-binding characteristics of a phytochelatin analogue: Implications for bioremediation."

- Molecular Nanotechnology II, La Jolla, Dec. 8-9, 1997, "Preparation and characterization of cysteine- and glutathione-capped novel nanocrystalline materials."

Consultative/advisory functions

- Reviewer for AFOSR, US Department of Agriculture
- Reviewer for scientific journals Biochemistry, Plant physiology, Insect Biochemistry, Comparative Biochemistry and Physiology etc.
- Reviewer for scientific grants submitted to New Zealand Foundation for Research, Science and Technology

Transitions

- The technical information concerning preparation of glutathione or PC-based CdS or ZnS semiconductor particles has been supplied to Dr. O. Mohideen of University of California,

Riverside for possible use in nanotechnology.

- The technical information concerning preparation of yeast cells loaded with CdS has been supplied to Dr. G. Bench of the Lawrence Livermore National Laboratory
- The technical information concerning the development of synthetic genes for phytoremediation has been supplied to University of California Toxic Substances Program.

New Discoveries/patents

- A genetically engineered yeast for overproduction of δ-amino-levulinic acid dehydratase has been designed.
- Methods have been devised for producing high-quantum yield semiconductor particles consisting of glutathione.
- Three disclosure statements have been filed during this period for the preparation of nanocrystalline semiconductors

Honors/Awards

None during this report period