was determined. Investigations of this variety on the aggregation of bombolitin III are currently being examined within our laboratories.

Registry No. L-Asp, 56-84-8; bombolitin I, 95648-97-8; bombolitin III, 95732-42-6.

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Phosphorylation of Iodopsin, Chicken Red-Sensitive Cone Visual Pigment†

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ABSTRACT: The amino acid sequence has been determined for the carboxyl-terminal 41 amino acids of chicken red-sensitive cone pigment, iodopsin. This sequence is distinct from but structurally homologous to that of other visual pigments. It contains a region rich in the hydroxy amino acids serine and threonine. In the related rod cell visual pigment, rhodopsin, such serines and threonines have previously been identified as sites for phosphorylation by rhodopsin kinase. Phosphorylation of photolyzed rhodopsin serves to terminate its ability to function in visual transduction as an activator of G-protein. We have purified and reconstituted both chicken rhodopsin and chicken iodopsin and shown them to be phosphorylated by bovine rhodopsin kinase. Chicken iodopsin has a K_m and V_{max} similar to but distinguishably different from that for bovine rhodopsin. These results, in conjunction with other data, suggest that visual pigments in cone cells, upon absorption of light, undergo functional processes similar to those of the visual pigments in rod cells.

Vertebrate rod cells mediate dim-light vision in black and white, whereas cone cells are responsible for color vision. Both rods and cones contain photoreceptor visual pigments that receive light energy and initiate the photoresponses. Rod cells have been more amenable to study than cones. Rod cell visual pigment, rhodopsin, and its interacting proteins have been prepared and characterized in some detail (Falk & Applebury, 1987). Although cone cells have many similarities to rod cells, they have distinct structural differences, operate at much higher light intensities, and have a different time constant of response (Pugh & Cobbs, 1986). In order to understand the functional differences between rod and cone photoreceptor cells, it will be necessary to prepare and study the properties of proteins in the cone cell visual transduction pathway.

The rod photoreceptor proteins are members of the family of receptor proteins that function via G-proteins (Applebury & Hargrave, 1986; Dohlman et al., 1987). After rhodopsin receives light it undergoes a change in conformation that

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enables it to bind the G-protein transducin and activates transducin by stimulating its binding of GTP. Transducin in the GTP-bound form then activates a cGMP-phosphodiesterase and the resulting hydrolysis of cGMP leads to a closure of cation channels in the rod cell plasma membrane and subsequent neural signaling (Stryer, 1986). Light-activated rhodopsin becomes phosphorylated by a specific cyclic nucleotide-independent kinase, rhodopsin kinase [reviewed by Kühn (1984); Palczewski et al. (1988a)]. This phosphorylation of rhodopsin is the ATP-dependent step that quenches cGMP-phosphodiesterase activation in rod cells (Sitaramayya & Liebman, 1983). This quenching of the visual excitation process is initiated by binding of a 48-kDa protein (arrestin) to phosphorylated light-activated rhodopsin, which leads to inhibition of the GTP binding to transducin (Wilden et al., 1986) or alternatively to the quenching of active phosphodiesterase directly (Zuckerman & Cheasty, 1988).

Rhodopsin kinase appears to bind to and phosphorylate at least two specific regions of photoactivated rhodopsin's cytoplasmic surface; its carboxyl-terminal region and its third cytoplasmic loop between helices V and VI [reviewed by Hargrave et al. (1988)]. Although the kinase can phosphorylate free peptides containing these sequences, it does so only poorly, suggesting multiple binding sites on the surface of the light-activated receptor (Palczewski et al., 1989). The bovine kinase recognizes rhodopsins from other species (Palczewski et al., 1988b).

The photoreceptor protein from the all-cone retina of a lizard has been shown to undergo light-dependent phosphorylation (Walter et al., 1986). With the exception of this observation, relatively little information is available concerning phosphorylation reactions in cone cells. Now with the availability of biochemical quantities of chicken rhodopsin and iodopsin in physiologically active form (Okano et al., 1989a; Fukada et al., 1989), it is possible to examine their phosphorylation by using the well-studied and purified bovine rhodopsin kinase. In the present paper we demonstrate that rhodopsin kinase effectively phosphorylates photolyzed iodopsin. Since the structure of the carboxyl-terminal region of chicken iodopsin shows presumptive phosphorylation sites similar to those in rhodopsin, this strongly suggests an underlying similarity between rod and cone cells with respect to the phosphorylation of their visual pigments. Recently, it has been demonstrated that functionally important sites, (i) a chromophore-binding site (Fukada et al., 1990) and (ii) a transducin-binding site (Fukada et al., 1989), in iodopsin are similar to those in rhodopsin. As cone cells are known to contain elements of the visual transduction cascade such as G-protein (Lerea et al., 1986) and cGMP-phosphodiesterase (Gillespie & Beavo, 1988), it is reasonable to propose that the basic processes of visual transduction in cone cells resemble those in rod cells.

Materials and Methods

Preparation of Visual Pigments and Urea-Washed Bovine ROS Membranes. Chicken iodopsin and rhodopsin were purified as described previously (Okano et al., 1989a) and reconstituted into L-α-phosphatidylcholine (PC,[1] from fresh egg yolk; Type XI-E, Sigma) liposomes at a molar ratio of PC to each visual pigment of 140–150 by dialysis (Fukada et al., 1989). Molecular weight for PC was taken as 860.

ROS were isolated from dark-adapted bovine retinas by sucrose gradient centrifugation and then washed with 5 M urea (Shichi & Somers, 1978; Palczewski et al., 1988b) for preparation of ROS membranes free of rhodopsin kinase activity (urea-washed ROS membranes).

Bovine rhodopsin was purified by concanavalin A–Sepharose 4B column chromatography using octyl glucopyranoside (Litman, 1982). The purified rhodopsin (1.6 mg/mL) was mixed with PC (dried from chloroform) at a molar ratio of PC to rhodopsin of 140–150. The sample was thoroughly dialyzed for 3 days against 3 × 4 L of 10 mM MOPS/NaOH buffer (pH 7.5) containing 140 mM KCl, 1 mM DTT, and 2 mM $Mg(OAc)_2$. The PC-reconstituted rhodopsin thus prepared was collected by centrifugation (20 min, 7800*g*). The pellet was washed twice with the same buffer. Finally, the PC-reconstituted rhodopsin was suspended in the MOPS buffer at 4 mg/mL.

Sequence Analysis of Iodopsin. All the procedures described below were performed in the light.

(*i*) *Digestion by CNBr.* Iodopsin (5–10 mg) purified in a CHAPS–PC system (Okano et al., 1989a) was delipidated with acetone and carboxymethylated with iodoacetic acid (Crestfield et al., 1963) after reduction under N_2 with 50 mM DTT in 0.5M Tris/HCl buffer (pH 8.5) containing 6 M guanidine hydrochloride and 3 mM EDTA. After stopping the reaction by the addition of excess (0.2 M) DTT, the mixture was dialyzed against 5 × 2 L of water, lyophilized, and dissolved in 70% formic acid. The carboxymethylated iodopsin was digested with 32 mg of CNBr at 20 °C for 24 h.

(*ii*) *Digestion by Achromobacter lyticus Protease I.* Purified iodopsin (0.1 mg) was dialyzed against 3 × 2 L of water, lyophilized, and then dissolved in 0.3 mL of 10 mM Tris/HCl buffer (pH 8.7) containing 8 M urea and 2 mM DTT. The iodopsin preparation thus obtained was mixed with 0.3 mL of water to lower the concentration of urea, to which 1.3 μg of C-Lys endopeptidase, *A. lyticus* protease I (Wako), was added. The mixture was incubated at 22 °C for 7.5 h, and then 1.3 μg of the enzyme was again added, followed by additional incubation at 30 °C for 20 h.

(*iii*) *Digestion by Staphylococcus aureus V8 Protease.* Purified iodopsin (0.3 mg in 10 mL) was dialyzed against 3 × 0.3 L of 10 mM Tris/HCl buffer (pH 8.0) containing 0.75% CHAPS, 20 mM NaCl, and 2 mM DTT, mixed with 10 μg of *S. aureus* V8 protease (Worthington) and then incubated at 25 °C for 10 h. Again, 10 μg of the protease was added, followed by incubation at 25 °C for 9 h.

(*iv*) *Digestion by Chymotrypsin.* Purified iodopsin (0.1 mg in 0.6 mL) was dialyzed against 3 × 1 L of 0.1 M NH_4HCO_3 (pH 8.0) containing 0.75% CHAPS and 0.1 mM DTT, mixed with 4 μg of TLCK-treated chymotrypsin (Type VII, Sigma), and then incubated at 22 °C for 25 h.

(*v*) *Sequence Analysis of Digested Fragments.* After the digestion of CNBr or one of the proteolytic enzymes, the digest was lyophilized, dissolved in a solvent (water:acetonitrile:TFA = 95:5:0.1), and centrifuged (90000*g*, 20 min) to remove insoluble materials. For isolation of peptides from the supernatant thus obtained, it was loaded onto a TSK gel ODS-120A (4.6 × 250 mm) or ODS-80T_M (6.0 × 150 mm) column (Toyo Soda) equipped with a HPLC system (Model 600E, Waters) and then eluted with a linear gradient of acetonitrile (concentration 5–95% in 0.1% TFA at a flow rate of 1 mL/min). Amino acid sequence of purified peptides was

[1] Abbreviations: CHAPS, 3-[(3-cholamidopropyl)dimethylammonio]-1-propanesulfonate; DTT, dithiothreitol; HEPES, *N*-(2-hydroxyethyl)piperazine-*N*′-2-ethanesulfonic acid; MOPS, 3-(*N*-morpholino)propanesulfonic acid; $PhMeSO_2F$, phenylmethanesulfonyl fluoride; PC, L-α-phosphatidylcholine from egg yolk; ROS, rod outer segment; TCA, trichloroacetic acid; TFA, trifluoroacetic acid; TLCK, *N*-*p*-tosyl-L-lysine chloromethyl ketone.

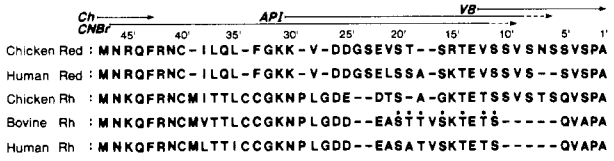

FIGURE 1: Primary structure of the carboxyl-terminal part of chicken iodopsin compared with that of other visual pigments. Sequence data for individual peptides obtained by digestion with CNBr, *A. lyticus* protease I (API), *S. aureus* V8 protease (V8), or chymotrypsin (Ch) are shown by arrows. Seven amino acid residues in bovine rhodopsin that are phosphorylated by rhodopsin kinase (Hargrave, 1982) are marked with asterisks. Visual pigment sequence data are from the following sources: human red pigment, Nathans et al. (1986); chicken rhodopsin, Takao et al. (1988); bovine rhodopsin, Ovchinnikov et al. (1982), Hargrave et al. (1983), and Nathans and Hogness (1983); human rhodopsin, Nathans and Hogness (1984). In this carboxyl-terminal part, the sequence of human green pigment is identical with that of human red (Nathans et al., 1986).

determined by a gas-phase automated sequencer (Model 477A, Applied Biosystems) and a phenylthiohydantoin (PTH) amino acid analyzer (Model 120A, Applied Biosystems).

Purification of Rhodopsin Kinase. Bovine rhodopsin kinase was prepared as described (Palczewski et al., 1989), except that 10 mM 1,3-bis[[tris(hydroxymethyl)methyl]amino]-propane buffer (pH 7.8) was substituted for Tris/HCl. The specific activity of rhodopsin kinase was 560 to 720 (nmol of P_i transferred/min)/mg of kinase.

Determination of Concentrations of Visual Pigments. Concentrations of visual pigments were determined from their absorption spectra by assuming the molar extinction coefficients (M^{-1} cm^{-1}) as follows: bovine rhodopsin, 40 600 at 498 nm (Wald & Brown, 1953); chicken iodopsin, 47 200 at 571 nm (Okano et al., 1989b). The molar extinction coefficient of chicken rhodopsin at 500 nm was assumed to be identical with that of bovine rhodopsin. The molecular weights of these pigments were taken as 40 000 (Hargrave et al., 1983; Takao et al., 1988).

Rhodopsin Kinase Assay and K_m Determination. Rhodopsin kinase activity was determined as described by Palczewski et al. (1988b). In order to obtain the maximum value of phosphorylation, the samples were sonicated for 3 min on ice. The phosphorylation obtained for the sonicated samples was approximately 25% higher than for the unsonicated one in the case of pigments in PC liposomes, but only a 5% increase in phosphorylation was observed for bovine rhodopsin in urea-washed ROS.

For the K_m determination, the visual pigment concentration was varied from $0.2K_m$ to $6K_m$ in the reaction mixture containing 100 μM [γ-^{32}P]ATP (200–350 cpm/pmol), and the reaction was carried out at 30 °C for 7.5 min in the light. Kinetic constants were determined from Lineweaver–Burk plots that were analyzed by linear regression. The K_m determinations were performed in 10 mM MOPS/NaOH buffer (pH 7.5) containing 140 mM KCl, 1 mM DTT, 2 mM $Mg(OAc)_2$, and 5% adonitol.

Results and Discussion

The carboxyl terminal sequence of chicken red-sensitive cone pigment, iodopsin, is presented in Figure 1. These data represent the only cone pigment amino acid sequence to be determined for a species other than human and directly from the protein rather than inferred from the sequence of DNA. The sequence is readily aligned with those of other vertebrate rod and cone pigments and is most similar to that of the human red and green cone pigments (Figure 1). One 22 amino acid stretch is identical with those of the human red and green cone pigments (22′–47′). However, there are as many as six positions of difference in the C-terminal 20 amino acids. Interestingly it is this region that contains the majority of the functionally important serine and threonine residues. It is these serines and threonines in the corresponding region of bovine rhodopsin that become phosphorylated by rhodopsin kinase (Hargrave et al., 1980; Thompson & Findlay, 1984). Chicken iodopsin is uniquely enriched in these hydroxy amino acids, having in its C-terminal 22 amino acids 11 such hydroxy amino acids, compared to 10, 10, 7, and 6 respectively for the human red/green cone pigment, chicken rhodopsin, bovine rhodopsin, and human rhodopsin. The significance of such variable proportions of carboxyl hydroxy amino acids is unclear. The human red/green cone pigment and chicken iodopsin share a unique feature in the 22′–47′ region: the lack of the Cys-(33′)-Cys(34′) sequence. All rod pigments whose sequences have been determined to date and nearly all other members of the class of G-protein-coupled receptors have one or two cysteines in this position (Dohlman et al., 1987). For rhodopsin, these cysteines have been shown to be palmitylated and are proposed sites of membrane attachment (Ovchinnikov et al., 1988). For example, for the β-adrenergic receptor, a single cysteine residue in this position is palmitylated, and this has been reported to be implicated in receptor function [see O'Dowd et al. (1989), but also see Karnik et al. (1988)].

When one compares the sequence of chicken iodopsin to those of human red/green cone pigment and the rhodopsins from chicken, bovine, and human (in Figure 1), one finds that of 47 positions, 17 are identical in all five pigments. Chicken iodopsin has four unique positions, different from those of the other four pigments. In addition to the 17 residues common to the five pigments, iodopsin has 24 positions (including deleted positions) identical with human red/green cone pigment, and 8, 5, and 3 positions identical with the rhodopsins from chicken, bovine, and human, respectively. This suggests that chicken iodopsin is more closely related to the human red and green cone pigments than to any of the rhodopsins.

In order to determine how the differences in visual pigment structure might be reflected in physiological function, we examined the phosphorylation of chicken rhodopsin and chicken iodopsin by bovine rhodopsin kinase and compared them with that for bovine rhodopsin. We found that the bovine rod cell kinase phosphorylates not only chicken rhodopsin but also chicken iodopsin. K_m values for both bovine and chicken rhodopsins in PC liposomes were essentially the same (Table I) and were higher than that for bovine rhodopsin in urea-washed membranes. The higher K_m for reconstituted bovine rhodopsin must in part be due to the fact that the cytoplasmic surface of some of the rhodopsin faces the inside of the vesicles and will not be accessible to the kinase.

Chicken rhodopsin is readily phosphorylated. However, the extent of phosphorylation of chicken iodopsin and chicken rhodopsin was 50% and 70%, respectively, that of bovine rhodopsin (Table I, Figure 2). This may be due in part to some particular requirements for the amino acid sequence in the phosphorylated region. However, the most striking observation from the study of phosphorylation of synthetic peptides from a variety of visual pigments was the wide variety of such sequences that are comparable substrates (Palczewski et al., 1989). It was concluded that a major factor in the phosphorylation of the visual pigments was the binding of the kinase to the photoactivated visual pigment (Palczewski et al., 1989). It appears that the binding of rhodopsin kinase involves a multisite attachment and comprises at least the carboxyl terminal and the second (between helices III and IV) and third (between helices V and VI) cytoplasmic loops (Palczewski et

Table I: Kinetic Parameters for Phosphorylation of Chicken and Bovine Visual Pigments[a]

visual pigment	K_m (μM)	V_{max} [(nmol/min)/mg]	extent of phosphorylation (mol of P_i/mol of visual pigment)
bovine			
ROS (urea washed)	4.1 ± 1	640 ± 120	6.8
Rh (in liposome)	12 ± 7	285 ± 147	3.9
chicken			
Rh (in liposome)	13 ± 6	360 ± 133	2.7
Iod (in liposome)	6.1 ± 3	110 ± 50	2.0

[a] The phosphorylation reactions were performed as described under Materials and Methods. The concentration of the visual pigments was varied between $0.2K_m$ and $6K_m$. The reaction was carried out under illumination for 7.5 min and then stopped with 10% TCA. The phosphorylated visual pigments were collected by centrifugation. Data are averages of 2–4 independent measurements.

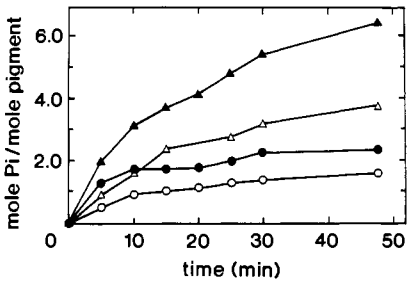

FIGURE 2: Time courses of phosphorylation of chicken and bovine visual pigments by bovine rhodopsin kinase. Bovine ROS washed with 5 M urea (closed triangles), PC-reconstituted bovine rhodopsin (open triangles), PC-reconstituted chicken rhodopsin (closed circles), or iodopsin (open circles) (each 2.7 μM pigment in 570 μL) was incubated with 10 μg of rhodopsin kinase and [γ-^{32}P]ATP (350 μM) in the dark or under illumination at 30 °C. At the indicated time 50 μL of the mixture was withdrawn and the reaction was stopped with 10% TCA. The membranes were collected by centrifugation and washed with 10% TCA. Finally, the pellet was dissolved in 100% formic acid and extent of the phosphorylation was determined by measurement of radioactivity. No appreciable phosphorylation was observed in the dark.

al., 1988b; Kelleher & Johnson, 1990). There are no sequence differences between chicken and bovine rhodopsin in the cytoplasmic surface loops implicated in the binding of rhodopsin kinase; the only differences are in the carboxyl-terminal region, which contains the phosphorylation sites. These differences may explain the lower extent of phosphorylation of chicken rhodopsin (Table I). Similar comparison with chicken iodopsin must await its complete sequence determination.

A goal of this kind of study is to make clear the differences in properties of the transduction cascade between rods and cones at the molecular level. Eventually one hopes to elucidate the biochemical basis of differences in their physiological responses to light. The current study, in which rhodopsin kinase was used instead of putative iodopsin kinase, represents an early effort aimed at understanding how the receptor potential of a cone cell recovers faster than that of a rod cell (Baylor & Nunn, 1982; Schnapf & Baylor, 1987). Even though the data obtained under the present experimental conditions are not always transferable to living cells, it is interesting to speculate that the lower K_m (higher affinity of photolyzed iodopsin for the kinase) and the lower extent of iodopsin phosphorylation might be able to explain in part the light response characteristic to cones. It is our future subject to test this hypothesis.

Registry No. Rhodopsin kinase, 54004-64-7.

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Double-Mixing Kinetic Studies of the Reactions of Methyl Isocyanide and CO with Diliganded Intermediates of Hemoglobin: $\alpha_2^{CO}\beta_2$ and $\alpha_2\beta_2^{CO}$ †

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ABSTRACT: Kinetics of the reactions of CO and methyl isocyanide with two diliganded intermediates of hemoglobin, $\alpha_2^{CO}\beta_2$ and $\alpha_2\beta_2^{CO}$, have been studied by double-mixing and microperoxidase methods. The valency hybrids were prepared by high-pressure liquid chromatography. The reaction time courses of ligand combination and dissociation with both of the ligands were biphasic, and in CO combination reaction the zero-time amplitudes of the two phases were independent of the protein concentration. In the presence of 2 M urea the reaction time course was clearly dependent on protein concentration, as the zero-time amplitude of the fast phase increased at lower protein concentrations. These two observations indicate that little dissociation of tetramers into dimers occurs in the absence of urea. Consistent with this, the kinetic data for the reactions of CO best fit a reaction model consisting of two tetrameric species not in rapid equilibrium with each other. Various considerations, however, suggest that the reaction model is more appropriately described as 2D $\rightleftarrows$ R $\rightleftarrows$ T. The reaction of triliganded species ($Hb_4(CO)_2Me_1$) with methyl isocyanide was monophasic, and the reaction model suggested a fast T $\rightleftarrows$ R structural change after the binding of the third ligand. Although the precise structural nature of the two species remains undefined, it is concluded that the biphasicity in the reactions of the two hybrids is characteristic of the diliganded species only and is independent of the nature of the ligand.

Valency hybrids $\alpha_2^{+CN}\beta_2$ and $\alpha_2\beta_2^{+CN}$, also known as symmetrical hybrids, have been extensively studied as models of diliganded hemoglobin intermediates. Cassoly and Gibson (1972) observed that the time course of CO binding to both of the hybrids was biphasic and the zero-time amplitudes of the two phases did not depend on the protein concentration. In their NMR studies of the valency hybrids, Ogawa and Shulman (1971) observed that at pH 7.3 these hybrids existed in two structures: one similar to deoxyHb and the other to oxyHb. These studies were made at heme concentrations of 2 mM. It was also observed that the lifetime of the two states was longer than 6 ms. Lack of concentration dependence of the relative amplitudes of the fast and slow phases suggests that the fast phase [$l' = (4 \pm 1) \times 10^6$ M^{-1} s^{-1}] cannot be assigned to the reaction of dimers. If the fast phase is assigned to the quaternary R structure and the slow phase to the quaternary T structure, then the interpretation of the kinetic and NMR data mentioned above would include slow rates of T $\rightleftarrows$ R transitions. This is contrary to the generally held view that the quaternary structural changes are fast. Contrary to the findings of Ogawa and Shulman (1971) and Cassoly and Gibson (1972), which suggest that the fast phase is tetrameric, the studies of Smith and Ackers (1985) indicate extensive dissociation into dimers of deoxy hybrids ($\alpha_2^{+CN}\beta_2^{deoxy}$ and $\alpha_2^{deoxy}\beta_2^{+CN}$). These researchers studies the rates of dissociation of deoxy hybrids into dimers by the haptoglobin method. Since this method is based on differences in the absorption coefficients of the R-state tetramer (or dimer) and the T-state tetramer, the results obtained in this study imply that the tetrameric form of deoxy hybrids is in the T state and is dissociated into dimers almost as much as normal oxyHb (oxyHb $k_{4,2}^1 = 1$ s^{-1}; deoxy hybrids $k_{4,2} = 0.8$–0.6 s^{-1}). Unfortunately, the presence of more than one tetrameric species may make it difficult to assign the k_{obsd} to $k_{4,2}$ (i.e., rate

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[1] Abbreviations: HPLC, high-pressure liquid chromatography; valency hybrids, $\alpha_2^L\beta_2^{+H_2O}$ or $\alpha_2^{+H_2O}\beta_2^L$; deoxy valency hybrids, $\alpha_2\beta_2^{+H_2O}$ or $\alpha_2^{+H_2O}\beta_2$; ferro or reduced hybrids, $\alpha_2^L\beta_2$ or $\alpha_2\beta_2^L$; $k_{4,2}$, rate constant for the dissociation of tetramer into dimers; Mp, microperoxidase; Me, methyl isocyanide; Bistris, 2-[bis(2-hydroxyethyl)amino]-2-(hydroxymethyl)propane-1,3-diol; IHP, inositol hexaphosphate; symbols R and T are used to denote fast and slow reacting species.

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