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Apple Four in Human Blood Coagulation Factor XI Mediates Dimer Formation†

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ABSTRACT: Human blood coagulation factor XI is a dimer composed of two identical subunits. Each subunit contains four apple domains as tandem repeats followed by a serine protease region. A disulfide bridge between Cys321 of each fourth apple domain links the subunits together. The role of Cys321 in the dimerization of factor XI was examined by mutagenesis followed by expression of its cDNA in baby hamster kidney cells. The recombinant proteins were then purified from the tissue culture medium and shown to have full biological activity. Normal recombinant factor XI was secreted as a dimer as determined by SDS-PAGE, while recombinant factor XI-Cys321Ser migrated as a monomer under these conditions. Gel filtration studies, however, revealed that each protein existed as a dimer under native conditions, indicating that the disulfide bond between Cys321 of each factor XI monomer was not necessary for dimer formation. The fourth apple domain (apple4) of factor XI was then introduced into tissue plasminogen activator (tPA) to investigate its role in the dimerization of other polypeptide chains. The fusion protein, containing apple4 (apple4-tPA), formed dimers as detected by SDS-PAGE and gel filtration. Furthermore, dimerization was specific to apple4, while apple3 had no effect on dimerization. These data further indicated that the apple4 domain of factor XI mediates dimerization of the two subunits and the interchain disulfide bond involving Cys321 was not essential for dimer formation.

Factor XI is a zymogen of a serine protease that participates in the intrinsic or contact phase of the blood coagulation cascade (Davie et al., 1991). Human factor XI is a glycoprotein that consists of two identical polypeptide chains held together by a single disulfide bond. Factor XI is activated by thrombin in the presence of a negatively charged surface (Naito & Fujikawa, 1991; Gailani & Broze, 1991), or by factor XIIa in the presence of high molecular weight kininogen and a polyanionic surface (Davie et al., 1979). Factor XIa is composed of two heavy and two light chains, which are held together by three disulfide bonds (McMullen et al., 1991). Each of the light chains contains the catalytic portion of the enzyme and is homologous to the pancreatic trypsin. Each of the heavy chains consists of 4 apple domains of 90 (or 91) amino acids (Fujikawa et al., 1986), and each apple domain has 3 characteristic disulfide bonds (McMullen et al., 1991). Several biological functions have been attributed to the apple domains. The first apple domains are involved in the binding of factor XI to high molecular weight kininogen (van der Graaf et al., 1983; Baglia et al., 1990), while the second apple domains are responsible for the calcium-dependent binding of factor XI to factor IX, its substrate (Sinha et al., 1985; Baglia et al., 1991). Each of the first and fourth apple domains contains an additional Cys residue. The two Cys residues at position 321 are linked together by a disulfide bond to form a homodimer.

In a previous study, the type III mutation of factor XI, in which Phe283 in the fourth apple domain was substituted by Leu (Asakai et al., 1989), was shown to be impaired in dimerization and secretion (Meijers et al., 1992). These results suggested that the fourth apple domains may be important in dimerization. In the present study, the role of Cys321 and the fourth apple domains in dimerization was examined.

Materials and Methods

Materials. Human factor XI and human plasma prekallikrein were purified according to previously published methods (Naito & Fujikawa, 1991; van der Graaf et al., 1982). The expression plasmids pZEM229R (Mulvihill et al., 1988) and ZpL7 (Johannessen et al., 1990) have been described. Prestained and [^{14}C]-labeled molecular weight markers were obtained from Bethesda Research Laboratories.

Construction of Factor XI Expression Vectors. The expression vector pZEM229R-XI was used for the expression of normal factor XI (Meijers et al., 1992). For mutagenesis of the cysteine residues, the human factor XI cDNA in M13mp18 was used (Meijers et al., 1992). Oligonucleotide primers PXI-M1 (AAGGACACCAGCTTTGAAGGA) and PXI-M2 (GCCCAAGCATCCAGCAACGAAGGG) were synthesized on an Applied Biosystems synthesizer and employed for mutagenesis of amino acid residues 11 and 321 from Cys to Ser. Mutagenesis was performed using T7-GEN (USB), by the method of Vandeyar et al. (1988). The mutations in the cDNA were confirmed by dideoxy sequencing, and the new constructs were then cloned into pZEM229R.

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The resultant plasmids were designated as pZEM229R-XI-C11S, pZEM229R-XI-C321S, and pZEM229R-XI-C11,321S.

Sequences of the cDNAs for each of the factor XI mutants were confirmed by dideoxy sequencing of the transfection plasmids.

Construction of Apple–tPA Expression Vectors. The tPA expression vector ZpL7 (Johannessen et al., 1990) was used as a template for the apple–tPA fusion protein construction. To facilitate the expression of tPA in cell culture containing normal fetal bovine serum and plasminogen, the active site of tPA was mutated from Ser to Ala using the primer MPS478A (GGGGCCTCCCGCATCGCCCTG). This plasmid was designated ZpL7(S478A). The *Bgl*II/*Xho*I fragment of ZpL7(S478A) coding for the finger and growth factor domains (amino acids 2–87) of tPA (Johannessen et al., 1990) was replaced by fragments encoding either the third (amino acids 181–271) or the fourth (amino acids 272–361) apple domain of factor XI. The polymerase chain reaction was used to generate fragments containing the third apple domain with the primer pair App5 (GCCAGATCT-GCTTGTATTAGGGAC) and App6 (GGCTCGAGTC-CCTCCTTTGATGCGTGGCACTGGGATGCTGTG), and the fourth apple domain with the primer pair App7 (GCCA-GATCTTTCTGCCATTCTTCA) and App8 (GGCTCGAGTCCCTCCTTTGATGCGTGGCTCATT-ATCCATTTT). Plasmids pZEM229-XI and pZEM229-XI-C321S were used as templates for the polymerase chain reactions, which were carried out in a thermal cycler from Perkin-Elmer as follows: a denaturation temperature of 94 °C (1 min) was followed by annealing at 45 °C (2 min) and extension at 72 °C (3 min) for 30 cycles. The final fusion constructs were designated as apple3–tPA–S478A, apple4–tPA–S478A, and apple4–C321S–tPA–S478A. The sequences of the fragments generated with the polymerase chain reactions were confirmed by dideoxy sequencing of the transfection plasmids.

Cell Culture, Transfection, and Purification of Recombinant Proteins. A thymidine kinase deficient baby hamster kidney cell line, BHK-570 (ATCC CRL 10314), was used as the host cell for the transfection and expression experiments. Cells were grown and transfected with an expression plasmid as previously described (Meijers et al., 1992). The purification procedure of the factor XI cysteine mutants from cell culture medium was the same as for normal recombinant factor XI (Meijers et al., 1992).

Cells transfected with tPA–apple constructs were grown in 100- or 150-mm plates until confluent, and serum–free collections were obtained after 24 h in serum-free medium containing 1000 units/mL aprotonin (NOVO, Denmark). Purification of tPA fusion constructs were performed with a monoclonal antibody to tPA (tPA-F2A1) bound to CNBr–Sepharose 4B (Pharmacia). The antibody was kindly provided by Dr. J. Selmer, Novo Nordisk. After application of the cell culture medium to the column, it was washed with 50 mM Tris, pH 7.4, containing 0.5 M NaCl. Elution was performed with 100 mM triethylamine, pH 11.5. Fractions of 1 mL were collected in 0.1 mL of 2 M Tris, pH 7.4, and dialyzed against 50 mM Tris, pH 7.4, 50 mM NaCl, and 0.02% sodium azide.

Gel Filtration Studies. Gel filtration studies were performed with purified rFXI and its cysteine mutants, or with concentrated culture media [25-fold concentrated with Centricon-30 (Amicon)] containing the apple–tPA fusion constructs. Samples were made 0.5 M in NaCl and applied to an FPLC Superose 12 column (Pharmacia), previously equilibrated with 50 mM sodium phosphate, pH 7.0, containing 0.5 M NaCl. The flow rate was 0.5 mL/min. The absorbance of the eluent was monitored at 214 nm, or fractions of 0.25 mL were collected.

Analytical Methods. Metabolic labeling of factor XI secreting cell lines was performed as described (Meijers et al., 1992). The radiolabeled secreted proteins were immunoprecipitated with a monoclonal antibody to factor XI [XI-5 (Meijers, 1988)] as previously described (Meijers et al., 1992).

Protein determinations were performed with the BCA-assay (Pierce) using bovine serum albumin as a reference. SDS–polyacrylamide gel electrophoresis was performed as described by Laemmli (1970). Factor XI activity was determined in a one-stage clotting assay employing factor XI-deficient bovine plasma (Tait & Fujikawa, 1987). Factor XI antigen was determined with an ELISA assay, using a monoclonal antibody to factor XI (XI-5) as primary antibody, and immunopurified rabbit antibodies to factor XI as secondary antibody (Meijers, 1988). Pooled normal human plasma (George King Biomedical) was used as reference for both the clotting and the ELISA assays, and contained, by definition, 1 unit/mL factor XI.

The tPA antigen concentration was determined with an ELISA assay as described by Brender and Selmer (1983). The ELISA was standardized against recombinant tPA, the concentration of which was determined by amino acid analysis.

Results and Discussion

When the primary sequence of human factor XI was established from the cDNA sequence, Cys11 and Cys321 were suggested as residues that might be involved in dimerization of the molecule by the formation of disulfide bonds (Fujikawa et al., 1986). Subsequent analysis showed that Cys11 of factor XI formed a disulfide bond with a free cysteine residue while Cys321 was involved in disulfide bond formation between the two monomeric subunits (McMullen et al., 1991). To evaluate further the role of these cysteine residues in dimer formation, Cys11 and Cys321 were changed to serine by site-specific mutagenesis. Normal factor XI (rFXI), single cysteine mutants (rFXI–C11S and rFXI–C321S), and the double mutant (rFXI–C11,321S) were inserted into the expression vector pZEM229R (Mulvihill et al., 1988) and expressed in stably transfected BHK cells. Factor XI antigen was readily detected in the culture medium of clones of each construct. The expression levels were determined with an ELISA assay using factor XI from normal human plasma as a reference. The levels of protein secretion were 0.17 ± 0.10 unit/mL for rFXI–C11S [number of independent clones tested (n) = 7], 0.09 ± 0.05 unit/mL for rFXI–C321S (n = 7), and 0.23 ± 0.13 unit/mL for rFXI-C11,321S (n = 7). These values were comparable to levels found for normal recombinant factor XI, which was 0.20 ± 0.09 unit/mL (n = 9) (Meijers et al., 1992). Also, Northern analysis performed with RNA from these cell lines showed that the mRNA levels for the cysteine mutants were comparable to the normal recombinant factor XI producing cell line (data not shown). These results indicated that the mutations at Cys11 and Cys321 have little if any effect on the transcription of normal factor XI mRNA, or with the secretion of the protein.

Metabolic labeling studies were then performed to demonstrate synthesis of recombinant factor XI by the stable cell lines. Metabolically labeled rFXI and cysteine mutants were characterized by SDS–PAGE (Figure 1). Without reduction, normal recombinant factor XI and rFXI–C11S migrated as dimers, while rFXI–C321S and rFXI–C11,321S were monomeric by SDS–PAGE. These results were in agreement

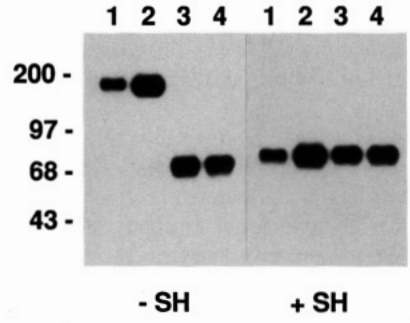

FIGURE 1: Characterization of secreted recombinant factor XI. Cells were metabolically labeled with [^{35}S]cysteine in cysteine-free medium. After 16 h, the secreted proteins were immunoprecipitated. The proteins were separated by SDS–PAGE on 7.5% gels in the absence (–SH) or presence (+SH) of a reducing agent. Lane 1, normal rFXI-secreting cells; lane 2, rFXI–C11S-secreting cells; lane 3, rFXI–C321S-secreting cells; lane 4, rFXI–C11,321S-secreting cells. The molecular masses of the marker proteins were indicated in kilodaltons.

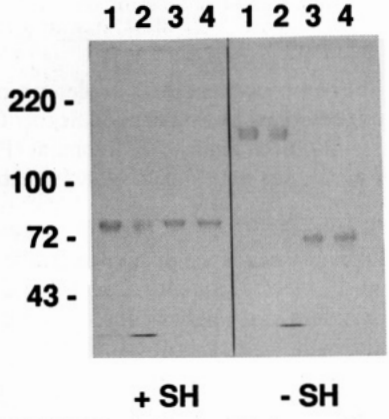

FIGURE 2: SDS–PAGE analysis of purified recombinant factor XI. Purified rFXI (lanes 1), rFXI–C11S (lanes 2), rFXI–C321S (lanes 3), and rFXI–C11,321S (lanes 4) were analyzed on 7.5% gels in the presence (+SH) or absence (–SH) of a reducing agent. The proteins were visualized by Coomassie staining. The molecular masses of the prestained markers are indicated in kilodaltons.

with those of McMullen et al. (1991), who showed that Cys321 was involved in dimer formation via disulfide bond formation. Substitution of this residue resulted in the elimination of a covalent disulfide bond and the appearance of monomeric factor XI upon denaturation by SDS without reduction. Upon reduction, however, all the factor XI mutants were composed of subunits that were identical in size (Figure 1).

The factor XI mutants were also purified from the cell culture medium using their affinity for a peptide derived from high molecular weight kininogen (Tait & Fujikawa, 1987). The purified proteins were homogeneous on SDS–PAGE (Figure 2). The specific clotting activities of the purified materials were 388 units/mg for rFXI-C11S, 333 units/mg for rFXI–C321S, and 349 units/mg for rFXI–C11,321S. These values were similar to, but somewhat higher than, the specific activity of 250 units/mg for normal recombinant factor XI (Meijers et al., 1992). These results infer that these cysteine mutations do not cause a significant change in the interaction of factor XI with factor XII, high molecular weight kininogen, and factor IX detectable in a surface-dependent clotting assay.

To determine whether the factor XI mutants with C321S were also monomeric in the native state, gel filtration studies were performed. Under the conditions used, plasma-derived factor XI and plasma prekallikrein were clearly resolved (Figure 3). The amino acid sequence of plasma prekallikrein

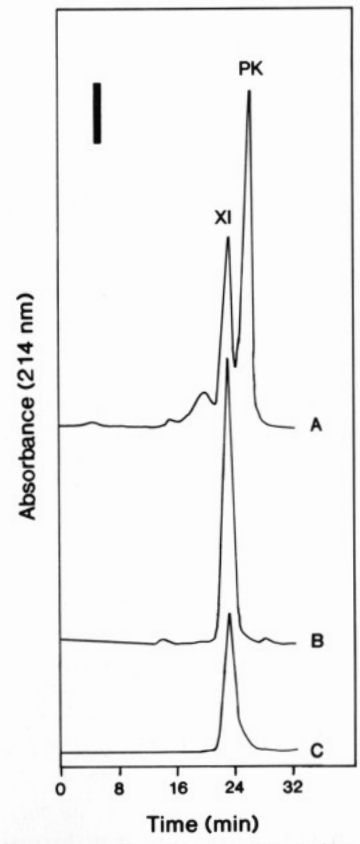

FIGURE 3: Gel filtration studies with recombinant factor XI. Purified recombinant factor XI and the cysteine mutants were applied to a Superose 12 column in 50 mM sodium phosphate, pH 7.0, and 500 mM NaCl, with a flow rate of 0.5 mL/min. The absorbance was read at 214 nm; the solid vertical bar is equivalent to an optical density of 0.01. (A) Plasma-derived factor XI (XI) and prekallikrein (PK); (B) normal recombinant factor XI; (C) rFXI–C321S.

is 58% identical to factor XI and also includes four homologous apple domains as tandem repeats followed by a serine protease domain (Chung et al., 1986). Plasma prekallikrein, however, is a monomer with a molecular weight of 80 000, which is the same as that expected for monomeric factor XI. rFXI and rFXI–C321S eluted at a position identical to that of plasma-derived factor XI, which is dimeric (Figure 3). These data indicated that these proteins also form dimers in solution, even in the absence of a covalent disulfide bond between the monomer subunits. Also, rFXI–C11S and rFXI–C11,321S eluted at the same positions as dimeric plasma factor XI (data not shown). The gel filtration results are consistent with the activity measurements that showed these mutants have similar specific activities. The results also showed that the interchain disulfide bond was not essential for dimer formation and suggested that specific recognition between two identical factor XI monomers was unaffected by the conversion of Cys11 and Cys321 to Ser.

Studies on a naturally occurring type III mutation of the factor XI molecule, in which Phe283 in the fourth apple domain was substituted by Leu, suggested that this mutation caused a drastic reduction in secretion of factor XI by interfering with the intracellular dimerization of the molecule (Meijers et al., 1992). Also, a mutation found recently in a Japanese patient (Nagoya II), in which Gly350 in the fourth apple domain was substituted by Glu, completely blocked intracellular dimerization and secretion of factor XI (R. Asakai, unpublished experiments). These data are consistent with the concept that apple4 may be involved in the initial recognition between the two factor XI subunits. The initial

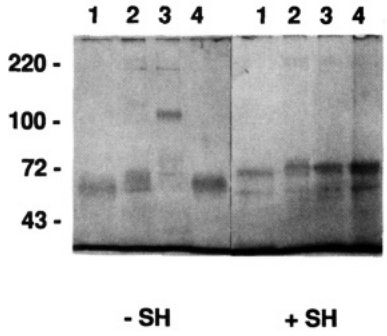

FIGURE 4: SDS–PAGE analysis of purified apple–tPA constructs. Purified apple–tPA fusion proteins were analyzed on 7.5% gels in the absence (–SH) or the presence (+SH) of a reducing agent. Lanes 1, tPA; lanes 2, apple3–tPA–S478A; lanes 3, apple4–tPA–S478A; lanes 4, apple4–C321S–tPA-S478A. The proteins were visualized by silver staining. The molecular masses of the prestained markers are indicated in kilodaltons.

recognition leads to the formation of a stable noncovalent dimer in which Cys321 in both subunits is positioned in close proximity for the subsequent formation of an interchain disulfide bond.

To demonstrate further that the dimerization process involves the apple4 domain, this domain was introduced into an unrelated protein tPA to investigate its effects on the physical properties of the fusion protein. In these studies, a modified cDNA for tPA (ZpL7) was used, in which convenient restriction enzyme sites were introduced (Johannessen et al., 1990). The active-site serine (Ser478) of tPA was first mutated to Ala to allow growth of transfected cells in normal serum containing plasminogen. The DNA coding for the finger and growth factor regions of tPA was then replaced by DNA fragments coding for either apple3 or apple4 of factor XI. The apple domains were followed by a short peptide sequence (PRIKGG) that contained a putative thrombin cleavage site. This region was introduced in order to retrieve and isolate, if necessary, individual apple domains from the modified tPA. The following apple–tPA fusions were constructed: apple3–tPA–S478A, apple4–tPA–S478A, and apple4–C321S–tPA–S478A. These constructs were transfected into BHK cells, and stable cell lines were isolated. The fusion proteins were readily detected in the culture media employing an ELISA assay for tPA. SDS–PAGE analysis of the secreted proteins showed that the construct yielding the apple4–tPA–S478A product was dimerized (Figure 4). In contrast, the stable cell line transfected with apple3–tPA–S478A resulted in the secretion of monomeric tPA as shown by SDS–PAGE (Figure 4). Also, mutation of the Cys321 to Ser in apple4 resulted in the formation of monomeric tPA on SDS–PAGE. After reduction, all the fusion proteins migrated as monomers (Figure 4).

To verify the physical appearance of these apple–tPA constructs, gel filtration experiments were then performed. These data showed that both apple4 constructs eluted in identical fractions as dimeric molecules (Figure 5) while apple3–tPA eluted at nearly the same position as native tPA. This indicated that apple4 by itself was sufficient to cause dimerization and that the disulfide bond between apple4 domains was not necessary. The apple4 domain was highly specific in causing dimerization, since other apple domains in factor XI (data not shown) and the homologous apple domains in prekallikrein do not cause this effect. Dimerization was apparently unaffected by the presence of up to 0.5 M sodium chloride, suggesting that the interaction between the apple4 domains is not entirely ionic in nature. The function of the

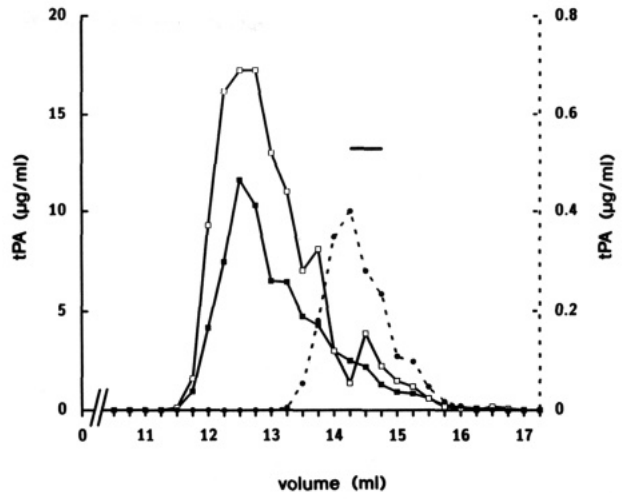

FIGURE 5: Gel filtration studies with apple–tPA constructs. Concentrated culture media were applied to a Superose 12 column in 50 mM sodium phosphate, pH 7.0, and 500 mM NaCl, with a flow rate of 0.5 mL/min. Fractions of 0.25 mL were collected and assayed for tPA with an ELISA. The horizontal bar indicates the elution position of tPA. The recombinant proteins are indicated by closed circles for apple3–tPA–S478A, closed squares for apple4–tPA–S478A, and open squares for apple4–C321S–tPA–S478A.

interchain disulfide bond is not clear at present.

The heavy-chain region of factor XI consists of four apple domains to which specific functions have been attributed. Recently, the binding of high molecular weight kininogen and factor IX to apple1 and apple2, respectively, has been demonstrated (Baglia et al., 1990, 1991). In the present study, the importance of apple4 in the dimerization of factor XI has been demonstrated. The fourth apple domain also seems to be involved in the secretion process, since the secretion of two naturally occurring mutations, type III and Nagoya II, was partially or completely blocked. An attractive hypothesis to explain why factor XI is dimeric might be that dimerization is necessary for the efficient intracellular processing and subsequent secretion of factor XI. If dimerization is blocked, such as in the Nagoya II deficiency, no secretion occurs. The secretion of the cysteine mutants as described in the present study was normal, since these mutations did not interfere with the dimerization per se, but only with the formation of the interchain disulfide bond. Further studies are necessary to provide proof for this hypothesis.

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Structures of Aspartic Acid-96 in the L and N Intermediates of Bacteriorhodopsin: Analysis by Fourier Transform Infrared Spectroscopy†

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ABSTRACT: The light-induced difference Fourier transform infrared spectrum between the L or N intermediate minus light-adapted bacteriorhodopsin (BR) was measured in order to examine the protonated states and the changes in the interactions of carboxylic acids of Asp-96 and Asp-115 in these intermediates. Vibrational bands due to the protonated and unprotonated carboxylic acid were identified by isotope shift and band depletion upon substitution of Asp-96 or -115 by asparagine. While the signal due to the deprotonation of Asp-96 was clearly observed in the N intermediate, this residue remained protonated in L. Asp-115 was partially deprotonated in L. The C=O stretching vibration of protonated Asp-96 of L showed almost no shift upon ^2H_2O substitution, in contrast to the corresponding band of Asp-96 or Asp-115 of BR, which shifted by 9–12 cm^{-1} under the same conditions. In the model system of acetic acid in organic solvents, such an absence of the shift of the C=O stretching vibration of the protonated carboxylic acid upon ^2H_2O substitution was seen only when the O–H of acetic acid is hydrogen-bonded. The non-hydrogen-bonded monomer showed the ^2H_2O-dependent shift. Thus, the O–H bond of Asp-96 enters into hydrogen bonding upon conversion of BR to L. Its increased hydrogen bonding in L is consistent with the observed downshift of the O–H stretching vibration of the carboxylic acid of Asp-96.

Bacteriorhodopsin (bR)[1] transports protons across the membrane by utilizing light energy absorbed by the retinylidene chromophore (Stoeckenius et al., 1979). The release of the proton from the membrane occurs at the L–M transition with the deprotonation of the Schiff base (Liu et al., 1990; Vârô & Lanyi, 1990b). The deprotonated Schiff base is then reprotonated by Asp-96 in the M–N transition (Holz et al., 1989; Otto et al., 1989; Butt et al., 1989). Proton uptake by the deprotonated Asp-96 from the cytoplasmic side completes the proton transport cycle. The L intermediate is thus a key intermediate, which determines the subsequent reactions for the utilization of light energy to produce a proton gradient.

Maeda et al. (1986) have previously argued that N (previously called L′) is similar to L in the resonance Raman spectrum. Vârô and Lanyi (1990a, 1991a) showed that L is in equilibrium with the preceding KL intermediate (Shichida et al., 1983) and also with the next M_1 intermediate (Vârô & Lanyi, 1990a). These reversible reactions between the intermediates are due to the small free energy differences among these three intermediates (Vârô & Lanyi, 1991c). The

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[1] Abbreviations: bR, bacteriorhodopsin; FTIR, Fourier transform infrared; BR, light-adapted form of bacteriorhodopsin.

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