Inhibition of Human Prolyl Hydroxylase as Common Biochemical Denominator of the Non-Sedative Effects of Thalidomide in Man

Hartmut Hanauske-Abel and Volkmar Günzler *

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The two main non-sedative effects of thalidomide (2-phtalimido-glutarimide) in man are the embryopathy, which finally forced termination of its use as a sedative, and the excellent efficacy in leprosy reactions, alone for whose prevention and treatment the drug is still available. Both effects can be explained on a molecular level by assuming that a non-sedative metabolite of thalidomide mediates inhibition of human prolyl hydroxylase, as suggested by steric considerations and correlation to known data. This metabolite may well be a suitable model compound for drugs designed for selective fibrosuppression and selective immunosuppression.

Introduction

The enzymatic post-translational conversion of genetically coded amino acid residues, standard for all proteins, to uncoded amino acid residues, specific for specialized molecules, is fundamental for structure and function of many proteins and hormones. Intentional intervention on certain modifications represents an essential part of pharmacology and is customary in therapy: The antithyroid drugs inhibit iodination of thyrosyl to 3- or 5-monoiodothyrosyl or 3,5-diiodothyrosyl residues within thyroglobulin [1, 2], the oral anticoagulants suppress vitamin K-dependent carboxylation of glutamyl to 4-carboxyglutamyl residues within Factors II, VII, IX, X [3–8].

We interpret the established non-sedative actions of thalidomide in man as an unintentional interference with the hydroxylation of prolyl residues (Pro) to *trans*-4-hydroxyprolyl residues (Hyp).

Hyp [9] occurs in the genetically distinct, partially sequenced collagens of type I, II, III [10], in less well-characterized collagenoid components of basement membranes ("type IV" [11]) and of human placenta [12], in the subcomponent C1q [13, 14] of the first component C1 (= C1q, C1r, C1s; "C1t" suggested) [15, 16] of complement [17], in elastin [18, 19], and in a protein of bone that contains 4-carboxyglutamyl residues, binds to hydroxyapatite via Ca^{2+} and could be a fragment of procollagen [20].

Abbreviations: β-Ala, 3-amino propionic acid; Pht, Phtaloyl-; Ag, Antigen; Ig, Immunoglobulin.

* *Present address:* Kantstraße 10, *D-3550 Marburg.*

Requests for reprints should be sent to H. Hanauske-Abel, Gladenbacher Weg 67, *D-3550 Marburg.*

In collagen molecules, the constant Hyp-content is the decisive parameter that determines the thermal stability of their classical triple helical, pepsin resistant *in vivo* structure: the thermal transition of the helical conformation in unhydroxylated molecules is 15 °C lower than in hydroxylated molecules, 24 °C versus 39 °C [21–25].

In C1q, a collagenase digestable, pepsin resistant sequence of 78 residues is arranged in 26 repetitive units of the collagen triplet type [13, 14, 143]. The similarity of C1q and collagen is also borne out by these proteins' ability to interact with DNA [26, 27] and by their competition for specific binding sites on sensitized erythrocytes and immune complexes [28, 29], or on human platelets [30, 31, 139]. Based on these chemical and biological findings, we assume that the triple helical structure within C1q [32] is also dependent on the Hyp-content under physiological conditions.

Both collagens and C1q can be produced and secreted by fibroblasts [33], and possess the repetitive triplet unit -X-Y-Gly- [10, 13]. The occurrence of Hyp is restricted to the N-terminal position next to glycine, *i. e.* to the sequence -X-Hyp-Gly- [10, 13].

The enzyme prolyl hydroxylase (E.C. 1.14.11.2) is responsible for the post-translational conversion of -X-Pro-Gly- to -X-Hyp-Gly- [34–46].

The enzyme belongs to a group of 2-oxoglutarate-coupled oxidoreductases occuring in man, animals, plants, microorganisms [36]; it needs a reducing agent, and its non-heme Fe^{2+} is directly involved in the catalytic action [39]. The enzyme utilizes molecular oxygen, introducing one atom into the substrate peptidyl proline at C4 in *trans* position, and the other one into 2-oxoglutarate at C2 leading to de-

carboxylation and formation of succinate [38]; its substrate specificity requires polypeptides of the physiological $(X\text{-Pro-Gly})_n$ structure, with $n = 1, 2 \ldots$ [38], and among non-physiological polypeptides, only $(X\text{-Pro-}\beta\text{-Ala})_n$-chains are hydroxylated, indicating a subordinate significance of C1 within the glycyl residue [42].

The enzyme occurs in various tissues [35, 40], and its subcellular location is restricted to the cisternae of the rough endoplasmic reticulum [38]; its activity is a marker for fibrogenesis: during periods of both physiologically or pathologically intensified collagen metabolism, *e. g.* embryonic morphogenesis [43], fibrosis of lung [44] and liver [46], marked increases precede and parallel the formation of connective tissue. The human enzyme has been purified from foetal skin and from a mixture of foetal tissues by affinity chromatography [41].

Hypothesis

We postulate that a non-sedative metabolite of thalidomide causes diminution of prolyl hydroxylation by direct inhibition of prolyl hydroxylase. The effect of the sedative parent compound (Fig. 1, **I**) on the activity of the enzyme has been studied without interpretable results [47].

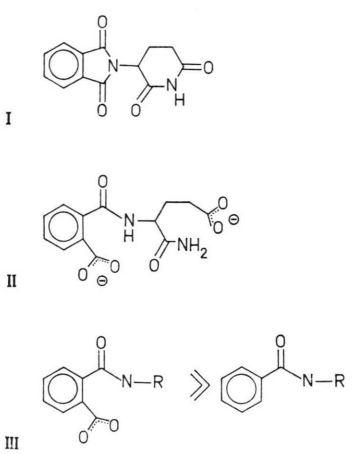

Fig. 1. **I** Thalidomide, 2-phtalimido-glutarimide. **II** 4-(*o*-carboxybenzamido) glutaramic acid anion, one of the hydrolysis products of thalidomide. **III** Structural elements which teratogenic substances of the thalidomide group have in common [79]. "≫" indicates differences in embryotoxicity: benzamide derivatives are less effective than *o*-carboxybenzamide compounds. A planar aromatic structure is indispensible for the embryopathic effects [78], 2-hexahydrophtalimido-glutarimide and 2-succinimido-glutarimide are *not* teratogenic [141]. Definite structure-function relations for R are not established [79].

The non-sedative hydrolysis product in question is 4-(*o*-carboxybenzamido) L-glutaramic acid (Fig. 1, **II**). It mimics conformation and configuration of the tripeptide Pro-Pro-Gly (Fig. 3) known to fulfill the minimal requirements of substrate specificity [38, 48–50] *.

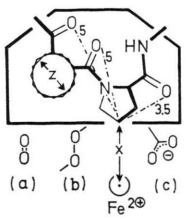

Fig. 2. Characteristics of binding site and catalytic site of prolyl hydroxylase relevant in this context. z measures about 2.5 Å and designates that part of the binding site which requires a planar shape in case of a ring structure integrated into the peptide backbone of the substrate; z refers to the distance of C atoms. The distance x between C4 within the appropriate prolyl residue and the iron atom was assumed to be 4 Å. x can easily be bridged by oxygen molecules certainly used in (a) and possibly occurring (b) during catalysis (a: molecular oxygen, O—O=1.2 Å; b: peroxide intermediate, O—O=1.5 Å), and by a carboxyl group affixed to C4 (c). The distance between C4 and the oxygen atoms of the carboxyl group is about 2.5 Å. — The proportions of binding and catalytic site, especially of a, b, c, x, and z, have been adhered to as far as possible. To illustrate the restrictions of two-dimensional reproductions, the distances between C4 and three peptidyl oxygen atoms as determined by model building have been inserted (unit: Å).

After binding, **II** is believed to form a complex with the enzyme-bound Fe^{2+} via its carboxyl group in the glutamic acid side chain (*cf.* Figs 2, 3), thus blocking the binding and catalytic site of the enzyme. The distance x between the iron atom and C4 within the appropriate prolyl residue was assumed to be $3.5\ \text{Å} \leqq x \leqq 4.5\ \text{Å}$ on grounds of the size of the oxygen substrate. x can easily be bridged by the carboxyl group mentioned above, provided that the positions of C3 and C4 in the prolyl and glutamyl residues are identical. The conformation of H-Pro-Pro-Gly-OH and Pht-Glu-NH_2 we assume on the basis of covalent- and van der Waals-radius is in agreement with the conformation of the hor-

* In an additional paper (V. Günzler, H. Hanauske-Abel, in preparation) we will provide details of an independent hypothesis concerning the catalytic mechanism of 2-oxoglutarate-coupled oxidoreductases: in brief, we propose that both the oxygen molecule and the 2-oxoglutarate molecule bind to a non-heme iron atom affixed to the protein. This produces an arrangement simultaneously facilitating decarboxylation and cleavage of the O—O bond. Our contention is in agreement with the known structural, stoichiometric and kinetic data of these enzymes.

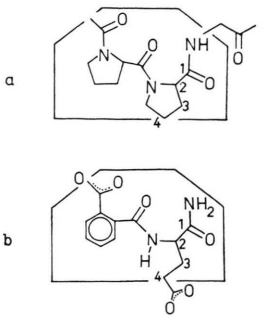

Fig. 3. a. Structure of a NH_2...-CO-Pro-Pro-Gly-...COOH segment of a collagen-like polypeptide chain at the binding site of prolyl hydroxylase. b. **II** arranged to fit the binding site of prolyl hydroxylase. The N-terminal aromatic ring is of the same size as the aliphatic ring in Fig. 3 a in spite of the different number of atoms due to the differences in bond lengths (C—C aliphatic, 1.54 Å; C—C aromatic, 1.39 Å).

mone H-Pro-Leu-Gly-NH_2, as proposed on energy calculations [51], as well as with the conformation of a simple model of collagen polypeptides, poly-L-proline II, as determined by X-ray diffraction [52, 53].

This hypothesis implies that the sequence -X-Glu-Gly-, since it also might be inhibitory, is not common in the natural peptide substrates of prolyl hydroxylase.

Collagen type I is the only large Hyp-containing protein whose primary structure is sufficiently well-known for examination of this deduction. Checking the amino acid sequence of its α1 and α2 subunits [10, 54], each one comprising more than 1000 residues, one finds 62 Glu residues occur in position X of the -X-Y-Gly- triplets, but only 9 in position Y. Asp residues, thought isofunctional with Glu residues in terms of protein-chemical features, exhibit a X/Y distribution of 19/21.

Discussion

I

In man, thalidomide causes a specific embryopathy that amounts to a phenocopy of the dominant Oram-Holt syndrome combined with the recessive Fanconi panmyelopathy [55, 56].

Collagens are integral parts of all connective tissue structures [57, 58]. They play a crucial role not only in scarring [137], in perfect parenchymal regeneration after injury [59] and in cell orientation [60], but also in cell differentiation and organ development during embryonic morphogenesis as demonstrated in vivo for ossification via an enchondral sequence and in culture for myogenesis [61–64]. Collagen molecules lacking the Hyp-dependent triple helical structure under physiological conditions, are not released at a normal rate by collagen synthesizing cells: in culture, the secretion of Hyp-deficient non-triple helical collagen precursors is markedly reduced or completely stopped by a conformation-dependent barrier within these cells [21, 38, 65–69]; in developing chick embryos, this retardation produces deformation of the limbs [70] and arrest of collagen accumulation [71]; in experimental cirrhosis, it diminishes the formation of collagen, and the lesser degree of fibrosis is accompanied by an improvement of liver function [72].

On the background of these findings, inhibition of prolyl hydroxylase and subsequent delay of collagen secretion can be expected to have distinct effects at least during periods of physiologically or pathologically intensified collagen metabolism.

Concerning embryonic morphogenesis, we anticipate the emergence of a complex clinical syndrome with various congenital anomalies comprising the entire width from agenesis of parts of the body to dysgenetic changes in their normal macroscopic and microscopic appearance. The extent and type of these malformations obviously depend on start, duration, dosage of drug administration to the mother. The non-hereditary alterations will affect supporting structures and parenchymal organs of the baby, involve both sexes in the same ratio, and follow a bilateral pattern of symmetry, as far as the enchondral osteogenesis in the limbs, *e. g.* the long tubular bones, is disturbed.

The thalidomide embryopathy [73, 74] conforms to this clinical syndrome.

The teratogenic action of thalidomide macroscopically and microscopically affects numerous parts of the body [73, 74] and is restricted to a short period of morphogenesis [74, 75]. In experimental animal, subperiostal osteogenesis histologically appeared normal, enchondral osteogenesis, however, was markedly disturbed during chondrogenesis and osteogenesis. Chondrocytes displayed unusual arrangement, intracellular granules and, generally, mesenchymal derivatives were particularly susceptible [76, 77]. The peptide substrate of prolyl hydroxylase bears a structural resemblance not only to the conformation of **II** but also to the configuration of embryopathic thalidomide analoga: the position of the indispensible planar structure in these substances (Fig. 1, **III**) corresponds to the nearly planar N-terminal ring of the mimicked -Pro-Pro-Gly- sequence (Fig. 3).

Concerning chronic diseases characterized by collagen accumulation in the affected tissues, we communicate a clinical observation in one patient suffering from pulmonary fibrosis (sarcoidosis of the lung): administration of the drug produced improvement of the pulmonary function. "Unter der Wirkung von K 17 verringerte sich das Sauerstoff-Defizit, so daß der Patient für längere Zeit auf die Sauerstoff-Flasche verzichten konnte" ** [80].

Due to the metabolite **II**, the thalidomide embryopathy unintentionally provides an experimental model of selective fibrosuppression in man testifying to the potentials of such pharmacologic treatment which is disastrous to the developing embryo and comparable to the oral anticoagulants as to teratogenity [81–83].

It is reasonable to use drugs that interfere with structure and secretion of collagens in all pathological conditions aggravated by fibrosis and scarring [84].

II

In man, "thalidomide is the most effective drug and in appropriate dosage can completely suppress ENL" [85], erythema nodosum leprosum.

ENL originates in patients fallen ill with the lepromatous form of leprosy, and by the end of the first year of anti-leprosy treatment, about 50% of such patients have experienced at least one episode of ENL [106–111].

Although it appears to be a tropical curiosity by West European standards, the histopathological findings in biopsy specimen of skin taken from acute ENL lesions document that its pathogenic mechanism is a prototype of immunological tissue damage: all the hallmarks of the Arthus reaction, an acute intense inflammatory lesion of skin that depends on antibody mediated complement activation [112] and can be initiated by the classical pathway [113–116], are evident in light and immunofluorescence microscopy [85, 117–119], and therefore acute ENL can be interpreted as a probe of complement activity.

The subcomponent C1q of the first component C1 of human complement links this system to the immunoglobulins and thereby triggers and trains the classical pathway [17].

Its activation has a causal connection to hereditary angioedema [86] and to the effects of certain allergens [87], participates in the immune response, *e. g.* in the rejection of allografts [88] or in the prodromal stage of acute viral hepatitis [89], and is a crucial factor in the pathogenesis of human immune complex diseases [90], *e. g.* systemic lupus erythematosus [91] or rheumatoid arthritis [92], as well as of experimental lesions in animals, *e. g.* the Arthus reaction and related conditions [93].

After antigen binding, IgG and IgM antibodies are enabled to interact with C1q [17, 94–96] at its six C-terminal globular regions [17, 97, 142]. This binding of immune complexes induces an allosteric rearrangement [17, 98, 99, 142], which makes C1r and C1s, bound over 100 Å away to the N-terminal collagen microfibril-like sector [139, 97, 142] trigger the classical pathway cascade [15, 17, 100–104, 140]. The structure-function relations of C1q are summarized in Fig. 4.

Selective destruction of the collagen-like parts within C1q molecules, the N-terminal sector and the

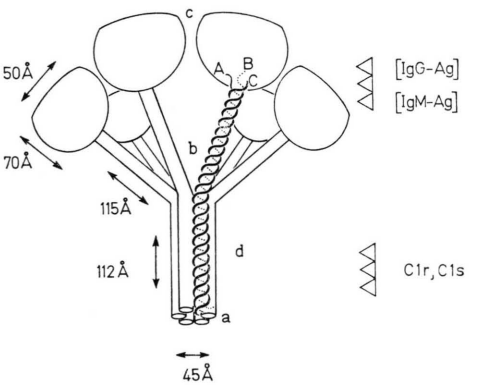

Fig. 4. Model of human complement subcomponent C1q [15, 17, 32, 97, 136, 139, 142, 143]. The collagen molecule-like substructure within one monomer of the hexamer is presented, consisting of three polypeptide chains (A, B, C) aligned in register. Besides a non-collagenous N-terminus of 2–8 residues (a), each monomer possesses an intermediate region of 78 residues forming a collagen molecule-like, triple helical strand (b), followed by a C-terminal noncollagenous region of 103–108 residues in globular arrangement (c). Side-by-side association of N-terminal triple helical monomer parts occurs covalently during dimer and non-covalently during hexamer formation, producing a collagen microfibril-like sector (d). This sector is linked by the unassociated C-terminal triple helical monomer parts, the connecting strands, to the globular regions [32, 136]. These interact with immune complexes via the Fc portions of IgG and IgM antibodies, whereupon a critical conformational change in the collagen microfibril-like sector (d) activates C1r, which in turn converts C1s to $\overline{C1s}$, triggering the complement cascade [17]. The allosteric rearrangement within d also is the prerequisite to activation by a C1r-independent interaction of C1s and C1q [15]. In this manner, the structure of C1q is fundamental for the release mechanism of the classical pathway of complement.

** K 17 (=thalidomide) decreased the oxygen deficit, so that the patient could do without administration of oxygen for longer periods.

connecting strands, abolishes the capacity to activate C1r and C1s and to trigger the classical pathway cascade, but does not abolish the capacity of immune complex binding [105, 144].

The action of **II** will selectively affect the collagen-like parts of C1q in a similar way. Provided that there is no conformational barrier within C1q synthesizing cells blocking the extrusion of the Hyp-deficient non-triple helical molecules, **II** will lead to the appearance of structurally and functionally impaired C1q in the serum, which we term "C1q **II**". We surmise that, due to their distorted conformation, these molecules lack the capacity to activate C1r and C1s. Consequently, a substitution of C1q **II** for C1q renders the first component useless and paralyses the classical pathway cascade, whose proteolytic activation yields fragments and culminates in membrane damage and cell death [17, 112]; these fragments mediate the chemotactic attraction and enhance the phagocytic ability at least of polymorphonuclear leucocytes, evoke a histamine-dependent edema and hyperemia reaction, and exert histamine-independent effects on the microvasculature, *e. g.* smooth muscle contraction. It is of preeminent importance that C1q **II** for these reasons will act as a selective suppressor of acute, classical pathway mediated inflammations: because of its strategic position [15], it will prevent the complement-dependent cytolysis and the formation of the strongly phlogogenic fragments.

In acute and persistent ENL, thalidomide has no antibacterial effect [120], its mode of action is unknown, its impressive efficacy established [106, 121–128].

Concerning treatment of *acute ENL*, no changes of the clinical symptoms occur during at least the first 36 hours after administration of the drug. On the end of the second day, various pathological findings start to ameliorate and on the following days return to normal.

There is a fall in fever, a reduction of the enlarged lymph nodes, a resorption of the subcutaneous nodules with decrease in size, erythema, tenderness, and even ulcerating lesions improve markedly. In biopsy specimen, polymorphonuclear leucocyte infiltration, hyperemia, edema, and other signs of acute inflammation disappear.

Upon withdrawal of thalidomide, relapse of ENL occurs after few symptom-free days: at the beginning, the recurring reactions are mild, but within days progress to the severe stage.

We argue that these effects are caused by disappearance and reappearance of subcomponent C1q, or of adequately functioning component C1, respectively.

Concerning treatment of *persistent ENL*, the uninterrupted use of thalidomide over a period of years does not cripple immunologic defence against bacteria, fungi, viruses, and does not render these thalidomide-dependent patients vulnerable to infections. Hence our hypothesis makes it necessary to suppose that a protracted selective *pharmacologic deficiency* of the classical pathway due to silenced C1, is not associated with an increased susceptibility to bacterial, fungal, viral infections. *Genetic deficiencies* of the early components of the classical pathway [129–132, 138] support this deduction.

Patients with these hereditary abnormalities "do not have an unusual susceptibility to infections, they illustrate how unimportant the classical pathway is for defense against the usual variety of Gram-negative and Gram-positive bacilli. *In vitro*, it is easily shown that bactericidal activity with unsensitized bacteria is normal, and with sensitized bacteria, markedly deficient" [133].

Due to the metabolite **II**, the thalidomide therapy of ENL unintentionally provides an experimental model of both selective immunosuppression and partial immunoprophylaxis in man, testifying to the potentials of such pharmacologic treatment which does not disarm the immune system of a patient and does not extradite him to bacteria, fungi, viruses, malignant tumors in complete contrast to the contemporary exercise of immunosuppression.

It is reasonable to use drugs that interfere with structure and function of C1 in all pathological conditions aggravated by activation of the classical pathway of complement [134].

We will not dwell here on further, less important evidence supporting this hypothesis. Systematic studies in drug design can be expected to produce specific inhibitors of other enzymes performing post-translational modifications, *e. g.* the lysyl hydroxylase [135], and to decide whether the *calculated interference with the function of specific molecules by disturbing the formation of their physiological structure without inhibiting precursor biosynthesis*, adds novel instruments to the tool-box of therapeutics.

We wish to express our deep esteem of the late Dr. Heide, who generously granted us unrestricted use of the library of the Behringwerke AG, Marburg,

and complete access to the computerized internal literature service of the Farbwerke Hoechst AG, Frankfurt. We are indebted to Prof. Karlson and Prof. Wiegandt for their help in preparing this manuscript; it was proofread by Dr. Nawrocki and typewritten by Mrs. Ballach. Mr. Weber, department head of the Zentralbibliothek der Medizin, Universitäts- und Staatsbibliothek, Köln, kindly met our wishes.

Addendum

Pilot studies at the Departments of Dermatology and Pathology, Royal University, Guardamangia/Malta, at the Department of Medical Microbiology, Johann Gutenberg Universität, Mainz/FRG, at the Department of Medical Biochemistry, University of Oulu/Finland, yielded preliminary results that seem to conform to our hypothesis:

In sera of ENL patients on thalidomide, C1q activity was reduced relative to the activity in sera of healthy persons and in serum of one patient suffering from a mild form of acute ENL; *in vitro*, **II** is an inhibitor of prolyl hydroxylase activity – whether **II** acts by the mechanism we suggest, is under scrutiny.

Thanks are due to Dr. Bonnici, Guardamangia, who supplied the ENL sera; to Dr. Pullicino, Guardamangia, who placed the serological laboratory at H.H.-A.'s disposal; to Prof. Loos,Mainz, who redetermined and affirmed the hemolytic complement activity and who measured the C1q activity of the ENL sera; to Prof. Kivirikko, Oulu, who tested the effect of synthetic II on the pure enzyme.

The extent of their cooperation, and the encouragement and assistance of those unnamed do not go without mention and cannot be overemphasized.

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