Human lactotransferrin: Molecular, functional and evolutionary comparisons with human serum transferrin and hen ovotransferrin[1]

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Summary. In this review article, human lactotransferrin is compared to human serum transferrin and hen ovotransferrin. For the first time the possibility of a 6-fold internal homology of the transferrins is raised: a scheme in which 6 domains are defined is reported; two of them with the highest homology seem to be implicated in the 2 iron binding sites of each transferrin. The location of the disulfide bridges of the 3 transferrins and of their prosthetic sugar groups is discussed: some not yet described half-cystine containing lactotransferrin peptides are indicated.

Transferrins constitute a family of homologous glycoproteins present in all vertebrate species. They consist of a single polypeptide chain of molecular weight close to 80,000 to which one or two carbohydrate groups are attached[5,25]. They also possess 2 metal binding sites each of which can bind a ferric ion (Fe^{3+}) together with a bicarbonate anion. The delivery of iron from transferrin to cells is mediated by the binding of transferrin $-Fe^{3+}$ complexes to specific cellular receptors[11,15]. Transferrins are primarily found in the sera of all vertebrates (serum transferrins), in bird egg-whites (ovotransferrins) and in almost all mammelian milks (lactoferrins or lactotransferrins). The complete amino acid sequences of human serum transferrin[19] (678 residues) and hen ovotransferrin (686 residues)[16,34] have been reported quite recently. Our group is currently involved in studies concerning human lactotransferrin and has described 75% of its sequence[23,24]. Lactotransferrin as well as serum transferrin and ovotransferrin present an internal homology[4,16,18,21,34]. Crystallographic[14] and partial proteolysis[32] experiments demonstrated that each half of the chain folds up into a largely independent compact domain (or lobe) which carries a specific metal binding site and it can be inferred that the disulfide bridges are located within the

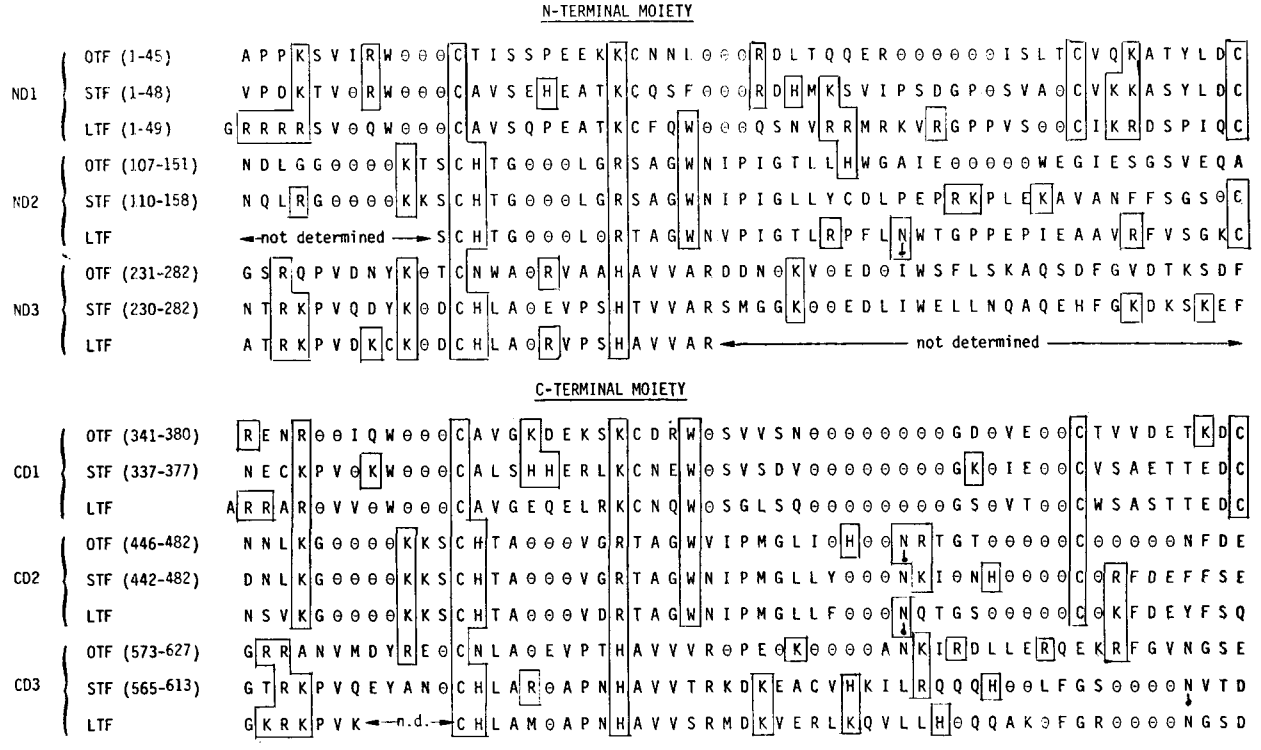

Figure 1. Suggested 6-fold internal homology of hen ovotransferrin (OTF)[16,34], human serum transferrin (STF)[18,19] and human lactotransferrin (LTF)[23,24]. 6 domains ND1 to ND3 and CD1 to CD3 are indicated. The important homologous residues (half-cystines, tryptophans and basic amino acids) are boxed (□); θ, deletion; the 1-letter amino acid abbreviation system is used; Ṇ, carbohydrate carrying asparagine residue.

Half-cystines of the N-terminal moiety of the 3 transferrins

Transferrin	Half-cystine residue number	Sequence	References	Disulfide bridge (No., see fig. 4)
OTF	10	R W C T I S		
STF	9	R W C A V S		1N
LTF	10	Q W C A V S	24	
OTF	20	K K C N N L R D		
STF	19	T K C Q S F R D		2N
LTF	20	T K C F Q H Q S	24	
OTF	36	I S L T C V Q K		
STF	39	P S V A C V K K		2N
LTF	40	P P V S C I K R	24	
OTF	45	L D C I K A I A		
STF	48	L D C I R A I A		1N
LTF	49	I Q C I Q A I A	24	
OTF	115	S C H T G L G R		
STF	118	S C H T G L G R		3N
LTF		S C H T G L R T	24	
OTF	160	F F S A S C V P		
STF	158	F F S G S C A P		4N
LTF		F V S G K C V P	*	
OTF	171	E Q K L C R		
STF	171	F P Q L C Q		5N
LTF		F P N L C R	*	
OTF	174	Q C K G		
STF	174	L C P G		4N
LTF		not determined		
OTF	182	K C A R N A P Y S G Y S		
STF	179	G C S T L D E Y F G Y S		5N
LTF		not determined		
OTF	197	F H C L K		
STF	194	F K C L K		3N
LTF		not determined		
OTF	228	E L L C L D G S R		
STF	227	E L L C L D N T R		6N
LTF		E L L C P D A T R	*	
OTF	242	T C N W A R		
STF	241	D C H L A E		6N
LTF		D C H L A R	24	

Additional half-cystines of STF and LTF

Transferrin	Half-cystine residue number	Sequence	References	Disulfide bridge (No., see fig. 4)
STF	137 (C)	I P I G L L Y C D L P		
OTF	134 (H)	I P I G T L L H W G A		10N
LTF	(P)	V P I G T L R P F L N	24	
STF	161 (C)	A P C A D G T D F		
OTF	163 (G)	V P G A T I E Q K		11N
LTF	(G)	V P G A D K G Q F	*	
STF	177 (C)	P G C G		
OTF	177 (D)	K G D P		11N
LTF	(X)	I G X X	24	
STF	331 (C)	N L R E G T C P E A		
OTF	336 (L)	S M R K D Q L T P S		10N
LTF		not determined		
STF	339 (C)	N E C K P V		
OTF	343 (N)	R E N R I Q		12C
LTF	(A)	R R A R V V	24	
STF	595 (C)	E A C V H K I L		
OTF	603 (K)	A N K I R D L L		12C
LTF	(L)	E R L K Q V L L	24	
STF	614,619(C)	T D C S G N F C L F		
OTF	622(K)627(E)	K S K F M M F E S Q		13C
LTF	(C)	S D C P D K F C L F	24	
LTF	(C)	K P V D K C K		
STF	238 (Y)	K P V Q D Y K		14N
OTF	239 (Y)	Q P V D N Y K	24	

Half-cystines of the C-terminal moiety of the 3 transferrins

Transferrin	Half-cystine residue number	Sequence	References	Disulfide bridge (No., see fig. 4)
OTF	348	I Q W C A V G K D E		
STF	345	V K W C A L S H H E		1C
LTF		V V W C A V G E Q E	24	
OTF	358	K S K C D R W S V		
STF	355	R L K C N E W S V		2C
LTF		L R K C N Q W S G	24	
OTF	371	V E C T V V		
STF	368	I E C V S A		2C
LTF		V T C W S A	24	
OTF	380	T K D C I I		
STF	377	T E D C I A		1C
LTF		T E D C I A	24	
OTF	405	A G V C G L V		
STF	402	A G K C G L V		9C
LTF		A G K C G L V	24	
OTF	421	Q C S K T D E R P A S Y		
STF	418	D C E Q T P A D G Y F A		8C
LTF		N C V D R P V E G Y L A	24	
OTF	454	G K K S C H T		
STF	450	G K K S C H T		3C
LTF		G K K S C H T	24	
OTF	478	G T C N F D		
STF	474	N H C R F D		7C
LTF		G S C K F D	24	
OTF	488	S E G C A P		
STF	484	S E G C A P		4C
LTF		S Q S C A P	24	
OTF	499	P P N S R L C Q L		
STF	495	K K D S S L C K L		5C
LTF		D P R S N L C A L	24	
OTF	502	L C Q G S G		
STF	498	L C M G S G		4C
LTF		L C T G X X	24	
OTF	513	K C V A S S H E K Y F G Y		
STF	506	L C E P N N K E G Y Y G Y		5C
LTF		not determined		
OTF	530	L R C L V E		
STF	523	F R C L V E		3C
LTF		F R C L A E	*	
OTF	570	L C T D G R		
STF	562	L C L D G T		6C
LTF		L C L D G K	*	
OTF	584	Y R E C N L A		
STF	576	Y A N C H L A		6C
LTF		X X X C H L A	24	
OTF	643	T K C L F K		
STF	636	T V C L A K		8C
LTF		T E C L A R	23	
OTF	671	L K T C N P		
STF	664	L R K C S T		7C
LTF		L R K C S T	23	
OTF	680	I L Q M C S F L		
STF	673	L L E A C T F R		9C
LTF		L L E A C E F L	23	

*Original results.

Half-cystine containing sequences of hen ovotransferrin (OTF)[16,34], human serum transferrin (STF)[18,19] and human lactotransferrin (LTF)[23,24]. The homologous residues are boxed: □, identical residues; ⬚, conservative changes. The 1-letter amino acid abbreviation system is used. N, carbohydrate carrying asparagine residue.

domains, none passing between them. The transferrin gene seems thus to have evolved from an ancestral gene[18] by duplication. The latter could be itself the product of a more ancient duplication: indeed, a weak 4-fold homology was observed in serum transferrin, suggesting that the molecule evolved from a protein of 1/4 of its present size[18].

In the course of our sequence studies devoted to human lactotransferrin, we raised for the first time the possibility of a 6-fold internal homology[24]. In the present paper we develop this hypothesis and propose that not only lactotransferrin but also serum transferrin and ovotransferrin can be organized into 6 domains. A comparative study of the 3 transferrins will then include sequence data and the localization of the half-cystine residues or disulfide bridges as well as of some biologically important amino acids, more particularly those involved in metal- or carbohydrate binding sites.

1. *Characterization of 6 domains (hexaplication) in the transferrins and sequence comparisons*

As our series of studies on human lactotransferrin proceeded, we became aware of a 6-fold occurrence of quite basic areas: the first one is situated at the N-terminal end of lactotransferrin and the others follow rather regularly roughly at every 110th amino acid residue. Thus, beside the previously demonstrated internal homology and the observation of a weak 4-fold homology, we propose now a 6-fold internal homology, suggesting that the molecule evolved from a protein of 1/6 of its present size. Lactotransferrin can be organized into 6 domains distributed 3 by 3 in the N-terminal (ND1 to ND3 domains) and C-terminal (CD1 to CD3 domains) moieties of the molecule. This 6-fold internal homology is exemplified in figure 1: not only the occurrence of rather basic N-terminal sequences for each domain but also the alignment of half-cystine, tryptophan and basic amino acid residues should, in particular, be pointed out.

The organization into 6 domains could be extended to human serum transferrin and hen ovotransferrin as also indicated in figure 1: each domain contains, again, 110±10 amino acid residues and the 6 domains of the 3 transferrins present many structural homologies (fig. 1).

2. *Comparative study of the half-cystine residues of the 3 transferrins*

When the nature of the amino acids conserved between the 6 domains of the 3 transferrins is analyzed, a large proportion appears to be of potential structural significance, such as the half-cystines, possibly reflecting the preservation of a similar 3-dimensional structure in the domains; indeed in the transferrins studied so far no cysteines occur: they are thus all involved in disulfide bonds.

Hen ovotransferrin possesses the smallest number of half-cystines (30 residues)[16,34]; 12 of them are situated in the N-terminal and 18 in the C-terminal moiety of the molecule (fig. 2). However, only 10 out of the 15 possible disulfide bond arrangements have been directly characterized thus far[13,34]; the remaining disulfide bonds must still be investigated. In human serum transferrin which contains 38 half-cystines[19] (fig. 2), 16 are found in the N-terminal moiety and 22 in the C-terminal one; here again the location of only 6 disulfide bonds out of 19 has been specified[9,19]. Human lactotransferrin, according to our sequence studies, contains at least 34 half-cystines[23,24].

In the 3 transferrins, the half-cystine residues are not randomly distributed; most of them are situated in quite homologous positions, even when the subdivision into 3 N-terminal and 3 C-terminal domains is considered. This is particularly true for the 30 half-cystines characterized in ovotransferrin which are found in homologous areas in serum- and lactotransferrins (table). The following disulfide bonds have been demonstrated in the domains a) of ovotransferrin[13,34]: 1 in ND1; 3, 4 and 5 in ND2; 6 joining ND2 and ND3; 1 in CD1; 3 in CD2; 7 and 8 joining CD1 and CD3; 9 joining CD2 and CD3; b) of serum transferrin[9,19]: 1 and 2 in ND1; 3 in ND2; 6 and 10 joining ND2 and ND3; 12 joining CD1 and CD3. These results as well as the internal homology detected in each transferrin and the close homologies established between the 3 transferrins suggest that the following 15 disulfide bonds seem to be conserved in the 3 molecules: 1N and 2N in ND1; 3N, 4N, 5N in ND2; 6N joining ND2 and ND3; 1C and 2C in CD1; 3C, 4C, 5C in CD2; 6C and 9C joining CD2 and CD3; 7C and 8C joining CD1 and CD3 (fig. 2).

Human serum transferrin contains 8 additional half-cystines when compared to ovotransferrin: 4 are located in the N-terminal moiety of the molecule (Cys-137, 161, 177 and 331) and 4 in the C-terminal moiety (Cys-339, 495, 614 and 619). These 8 half-cystines might correspond to 4 additional disulfide bonds: 10N which joins domains ND2 and ND3; 11N situated in the domain ND2 and probably contributes to its stabilization; 12C joining domains CD1 and CD3 and contributing to transform the C-terminal moiety into a rather compact region; 13 situated in the domain CD3.

At the present stage of our study, 4 additional half-cystines occur in lactotransferrin instead of 8 in serum transferrin when compared to ovotransferrin; they might correspond to 2 cystine bonds: one seems homologous to bond 13C of serum transferrin (fig. 2). The additional disulfide bonds 10N, 11N and 12C of serum transferrin when compared to ovotransferrin do not have a counterpart in lactotransferrin.

3. *Localization of the amino acids involved in the iron binding sites*

If some conserved residues of the transferrins might have a structural significance, some others can be expected to have a functional one. The stereochemical restrictions on the arrangements of liganding groups in the iron binding sites would result in a conservation of the residues involved in these binding sites. It is generally thought that each iron binding site contains 3 tyrosyl[3,31], 2 histidyl[17,20] and also tryptophan[30] residues; the concomitantly bound bicarbonate anion[29] may be held electrostatically to an arginyl side group[27].

Which domain(s) fulfilled these requirements? Taking into account once more the internal homology and the homologies between the 3 transferrins, it was possible to eliminate domains ND1/CD1 and ND3/CD3: indeed in the first 2, only 2 conserved tyrosines and 1 conserved tryptophan were characterized; in the 2 other domains the conserved residues included only 1 tyrosine, 1 histidine and 2 arginine residues. The 2 homologous domains ND2 and CD2, however, can be considered as serious candidates for the 2 iron binding sites; each of them possesses the following conserved residues (fig. 3): 2 tyrosines, 1 tryptophan, 1 histidine and 1 arginine. In the scheme similar to that proposed by Williams et al.[34] for hen ovotransferrin, we indicate (fig. 4) the 6 domains which we characterized in lacto- and other transferrins, and more particularly the ND2 and CD2 domains: they can clearly be defined by the occurrence of disulfide bonds 3N and 3C and the 5 above mentioned conserved amino acids are thus brought together. Not only the primary but also the secondary structures of this area of the 3 transferrins present a high degree of homology: the 3rd conserved tyrosine residue implicated in the iron binding site might be situated in the D1 domain and is in fact, according to the secondary structure, in close contact with the other important residues. The same observation could be made with respect to the 2nd conserved histidine residue situated in the D3 domain.

4. *Localization of the glycans in the transferrins*

It is well known that the tripeptide Asn-X-Thr/Ser[26] represents the code sequence for N-glycosidically

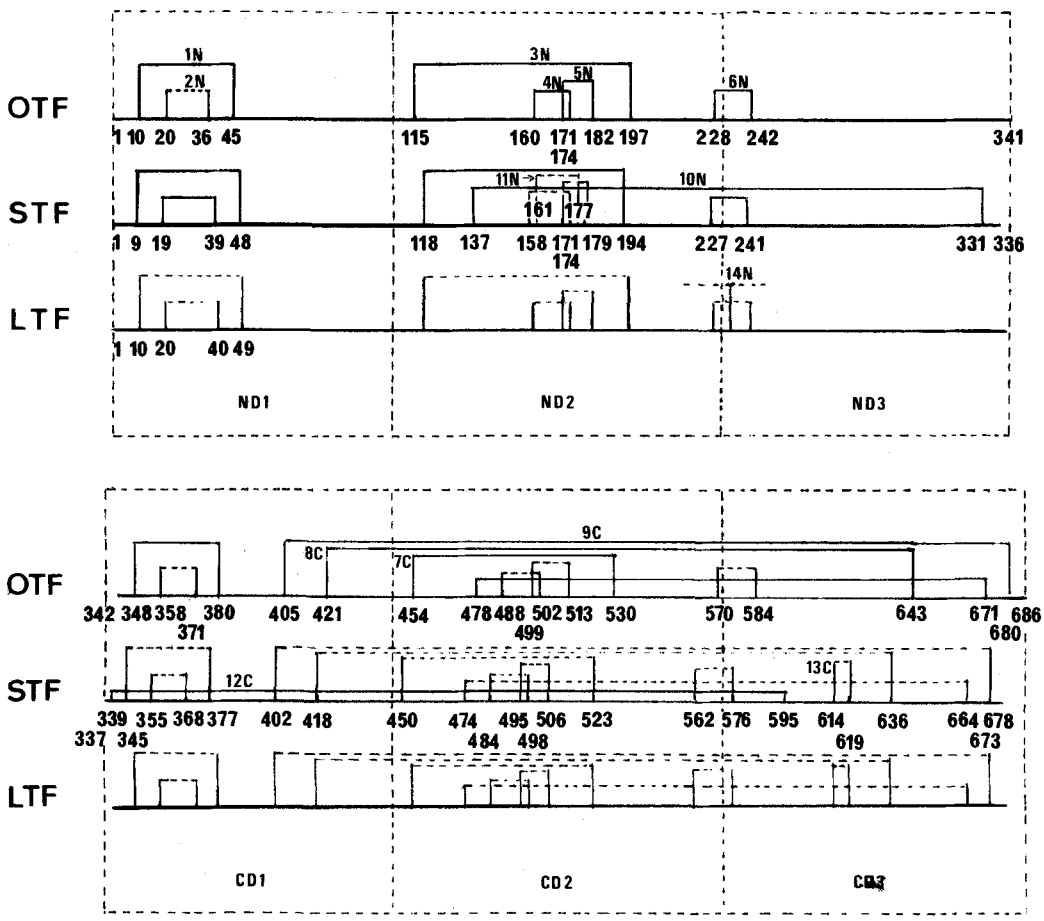

Figure 2. Diagram of the half-cystine positions of 3 transferrins: OTF[16,34], STF[18,19] and LTF[23,24]. Not yet determined disulfide bridges are indicated by dashed lines. The 6 transferrin domains are termed ND1 to ND3 and CD1 to CD3.

linked sugar groups. An investigation concerning the occurrence of this tripeptide in each domain of the 3 transferrins was carried out. In hen ovotransferrin one code sequence was located in the CD2 domain and 2 others in the CD3 domain, but only the former was glycosylated (fig. 4). In human lactotransferrin, 4 code sequences were characterized in the domains ND2, CD1, CD2 and CD3. According to our sequence studies, only 2 of them situated in the domains ND2 and CD2 were glycosylated (fig. 4). Human serum transferrin contained 2 code sequences in the domains CD2 and CD3: both of them were glycosylated (fig. 4). The carbohydrate-carrying asparagine residue of the code sequence is generally located in a special conformation called β-turn[6,7] and in a hydrophobic environment[8]. The secondary structures and the hydrophobicities of the areas surrounding the above mentioned 4 non-glycosylated tripeptides Asn-X-Ser/Thr have been established. There is a high probability that the asparagine residue of the tripeptide of lactotransferrin situated in the CD3 domain and asparagine 618 of ovotransferrin are in a β-turn conformation and the remaining secondary structure of the surrounding sequences is identical to the homologous region of serum transferrin[23] where the asparagine is glycosylated. The absence of glycosylation of the second asparagine residue of ovotransferrin situated in the CD3 domain (Asn 678) can be explained by the presence of a proline residue between the asparagine and serine residues[28]. Finally the asparagine residue

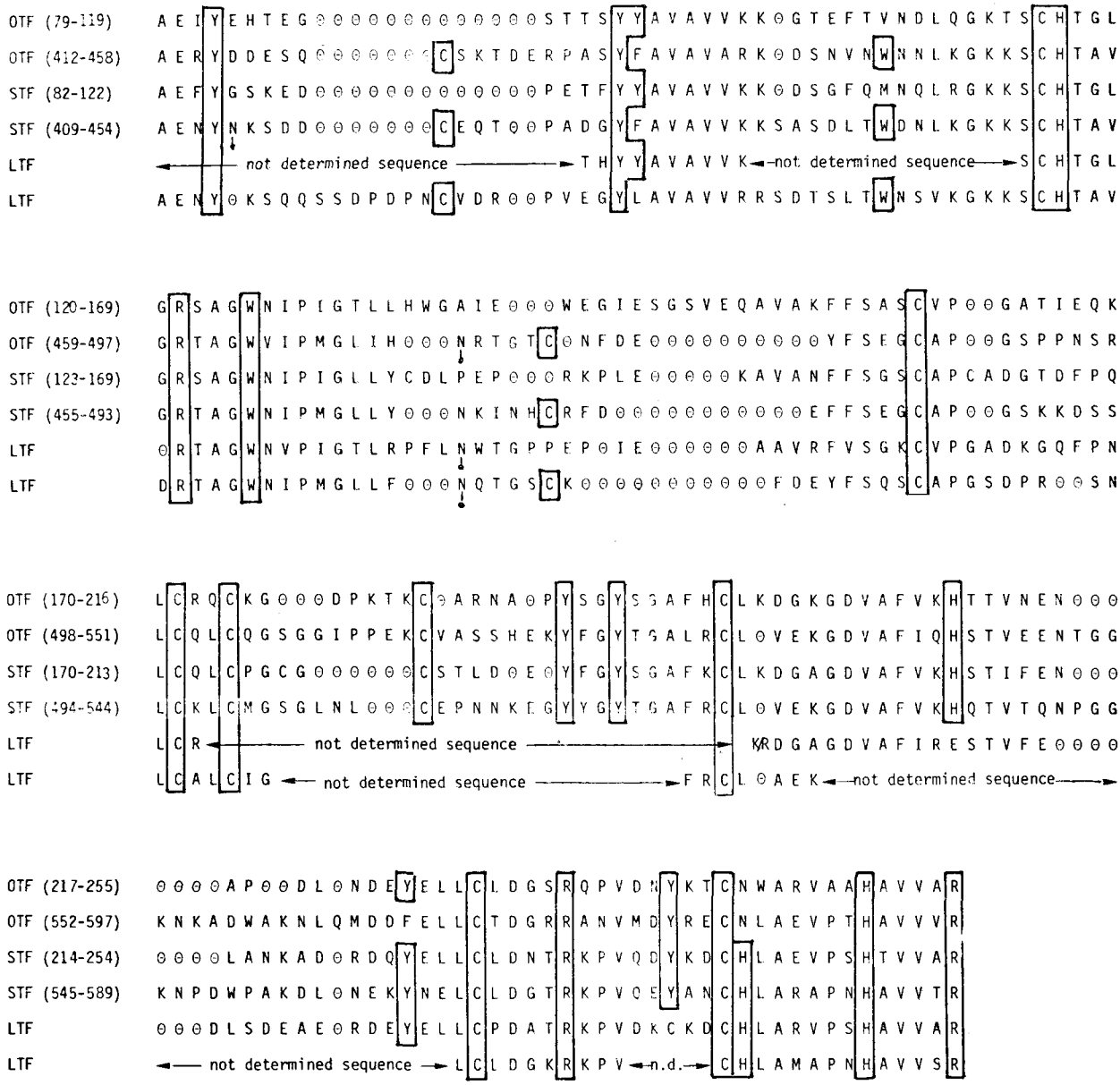

Figure 3. Long transferrin sequences (OTF[16,34]), (STF[18,19]), (LTF[23,24]) containing amino acids which might be involved in iron binding sites: these residues are boxed. θ, deletion; $\underset{\downarrow}{N}$, carbohydrate carrying asparagine residue.

of lactotransferrin encountered in the CD1 domain is situated in a sequence whose probability to adopt a β-turn is too low[10].

These results demonstrate that the glycans are preferentially found in the domains ND2, CD2 and CD3. The CD3 domain of the 3 transferrins contains the code sequence but only in human serum transferrin the tripeptide is glycosylated.

Discussion

The results discussed in this article and most particularly in the 1st and 3rd parts favor the existence of 6 homologous domains (hexaplication) in transferrins; the possibility exists that the 6-segment structure might have been generated by a series of duplications of an ancestral 110-residues unit; a possible candidate for this ancestral 110-residues unit might be the metal-carrying domain ND2 (or CD2) which is the one most conserved. Homology between domains ND2 and CD2 of ovo- and serum transferrins is around 70% and drops to around 40% between ND1 and CD1 or ND3 and CD3. Quite recently it has been reported[12] that a ribosomal protein, the 557-residues long S1 fraction from *E. coli*, is also made up of 6 homologous segments, each of which is approximately 88 residues in length; it has further been claimed that the fundamental repeat unit in the S1 protein can be reduced to 44 residues; in the transferrins it does not seem possible to reduce the 110-residues unit.

Further data emerging from this study permit a more precise definition of some other current problems in the transferrin field – particularly those concerning the metal binding areas. We have inferred that the conserved residues, among them the tyrosine, histidine, arginine and tryptophan residues of the D2 domains, might be involved in iron binding sites. More particularly, 2 of the 3 tyrosyl residues implicated in each binding site belonged to ND2 and CD2 domains when the third was located in D1 domains. Recently, after chemical modification of the tyrosines of iron saturated hen ovotransferrin by nitration with tetranitromethane, Williams[33] identified 8 protected tyrosine residues implicated in metal binding sites: 2 of them were present in D2 domains and the 2 others in D1 domains: this result was in accordance with our data mentioned above. Furthermore we recently established a structural relatedness between human lactotransferrin and human ceruloplasmin[22]. Sequences homologous to those containing the copper-binding sites in *Pseudomonas aeruginosa* azurin, *Anabaena variabilis* plastocyanin and probably ceruloplasmin were characterized in the transferrins: they were situated in the ND3 and CD3 domains; thus the copper and iron binding sites seemed to be located in different parts of the transferrin molecules.

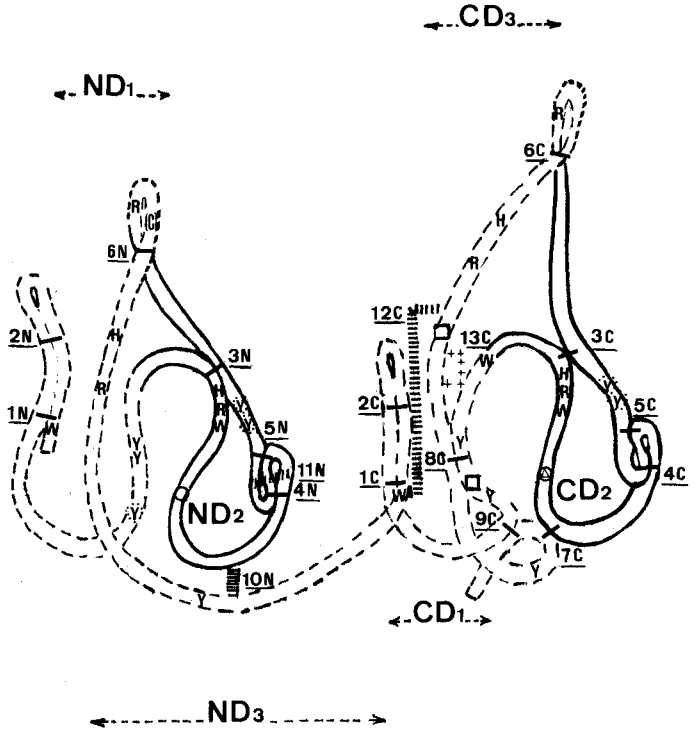

Figure 4. A transferrin molecule model for LTF, STF, OTF with disulfide bridges (1 to 13)[34], location of the 6 domains (ND1 to ND3 and CD1 to CD3) and indication of the conserved residues which might be implicated in the iron binding site(s); the 1-letter amino acid abbreviation system is used: R, arginine; H, histidine; W, tryptophan; Y, tyrosine; Y, not yet determined tyrosine; [C], half-cystine residue located only in lactotransferrin. ○, □, △, carbohydrate-carrying asparagine residue in LTF[24], STF[18], OTF[34], respectively. ///, disulfide bridges present only in serum transferrin; +++, disulfide bridges present in serum transferrin and lactotransferrin.

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Short Communications

Further caulerpenyne-like esters from the green alga *Caulerpa prolifera*[1]

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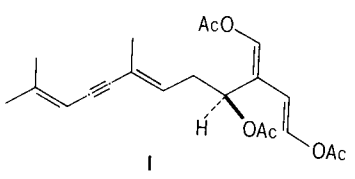
Summary. From a further investigation of the extractive of the green marine seaweed *Caulerpa prolifera,* we isolated **III**, which, on the basis of chemical and physico-chemical data, proved to be a dihydroderivative of caulerpenyne with an acetoxy group substituted by fatty acid residues.

Two previous reports from this laboratory[2,3] describe the isolation from *Caulerpa prolifera,* a green marine seaweed widely distributed in Mediterranean waters, of a linear sesquiterpenoid, caulerpenyne (**I**), and of furocaulerpin (**II**), biogenetically related to (**I**).

Structurally related sesquiterpenoids and diterpenoids with antimicrobial and antifeedant activities[4,5] were also found in other species of Chlorophyceae belonging to the same order (Siphonales), and this could have a chemotaxonomic significance. The biological properties of this class of natural compounds prompted us to investigate minor constituents of *C. prolifera* and in this paper we describe the isolation and structure elucidation of further caulerpenyne-like esters from this alga.



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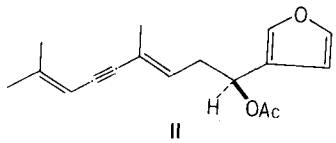

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