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Evidence for interactions between the 30 kDa *N*- and 50 kDa *C*-terminal tryptic fragments of human lactotransferrin

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Gel filtration of a mild tryptic digest of diferric human lactotransferrin carried out in presence of 10% (v/v) acetic acid led to the isolation of two fragments, an *N*-terminal tryptic fragment having an M_r of 30000 and a *C*-terminal tryptic fragment having an M_r of 50000 [Legrand, Mazurier, Montreuil & Spik (1984) Biochim. Biophys. Acta **787**, 90–96]. Both fragments possess a degree of organization lower than that of the native protein, as shown by the decrease of about 30% of the α-helical content observed by c.d. The two fragments are able to re-associate in neutral solutions, as shown by the isolation, by gel chromatography, of a re-associated 80 kDa *N,C*-tryptic complex having the chromatographic behaviour of the native lactotransferrin. Computer-based comparison of the measured c.d. spectrum of the mixture of *N*-tryptic and *C*-tryptic fragments (molar ratio 1:1) with the spectrum calculated by assuming one molecule of each fragment, shows that the α-helix content of lactotransferrin is restored. These results strongly suggest the existence of non-covalent and reversible interactions between the two lobes of lactotransferrin. In addition it was demonstrated that short peptide segments (residues 19–24, 45–58 and 264–276) are involved in the secondary-structure modifications referred to above.

INTRODUCTION

The bilobal structure of the transferrins, each lobe containing one iron-binding site, is generally accepted on the basis of the crystallographic data reported by Gorinsky *et al.* (1979) for rabbit serotransferrin and by Abola *et al.* (1982) for hen ovotransferrin. This view is reinforced by the comparative study of amino acid sequences of hen ovotransferrin (Williams *et al.*, 1982), human serotransferrin (MacGillivray *et al.*, 1983) and human lactotransferrin (Metz-Boutigue *et al.*, 1984) as well as by the nucleotide sequence of the cDNA of hen ovotransferrin (Jeltsch & Chambon, 1982) and human serotransferrin (Yang *et al.*, 1984; Uzan *et al.*, 1984; Park *et al.*, 1985). In addition, the two lobes can be isolated by limited proteolysis of the monoferric and/or diferric forms of transferrins (Bezkorovainy, 1980).

The bilobal feature of transferrins raises the question of the existence of interactions between the two lobes. The first approach to the problem was made by thermal-denaturation studies of ovotransferrin, which have demonstrated the existence of co-operative interactions between the two lobes (Evans *et al.*, 1977). This result has been recently corroborated by partial tryptic cleavage of iron-saturated ovotransferrin into two fragments still non-covalently bound (Ikeda *et al.*, 1985; Evans *et al.*, 1985).

With regard to human lactotransferrin (also called 'lactoferrin') (for recent review, see Montreuil *et al.*, 1985), we have previously shown that mild digestion with trypsin under non-denaturing conditions cleaves the diferric molecule at residue 281, giving an *N*-terminal tryptic fragment (Nt-fragment; M_r 30000; residues 3–281) and a *C*-terminal tryptic fragment (Ct-fragment; M_r 50000; residues 282–703) (Legrand *et al.*, 1984). A second mild treatment of the purified *N*-tryptic fragment with trypsin leads to the liberation of small peptides and of a so-called 'N2-glycopeptide' (M_r 18500; residues 91–257) in which the *N*-terminal saturated iron-binding site is located (Spik *et al.*, 1983; Legrand *et al.*, 1984). These findings can be seen to be the result of the protection, against proteolytic attack, of the *N*-terminal lobe by the *C*-terminal lobe, and they prompted us to study the inter-domain contacts within the lactotransferrin molecule.

MATERIALS AND METHODS

Gel-filtration chromatography of the tryptic digest of lactotransferrin

Pure diferric human lactotransferrin was obtained as previously described (Spik *et al.*, 1982). Limited cleavage of diferric lactotransferrin [1% (w/v) solution in 0.1 M-Tris/HCl buffer (pH 8.2)/0.025 M-$CaCl_2$] was performed with trypsin (Worthington Biochemical Corp., Freehold, NJ, U.S.A.) (enzyme/substrate ratio 1:50) at 37 °C for 4 h. A 10 ml portion of the digest was further submitted to gel-filtration chromatography on a column (5 cm × 100 cm) of Sephadex G-75 or Bio-Gel P-60, either under non-denaturing conditions (0.1 M-NH_4HCO_3) or under denaturing conditions [0.1 M-NH_4HCO_3 containing 4 M-urea or 1 M-KCl or 15% (v/v) ethanol]. The iron-free *N*- and *C*-tryptic fragments were isolated by gel-filtration chromatography of 30 ml of the digest on a column (5 cm × 100 cm) of Bio-Gel P-60,

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equilibrated in 10% (v/v) acetic acid as described by Legrand *et al.* (1984). The N2-glycopeptide was purified from the tryptic digest of iron-saturated *N*-tryptic fragment by gel-filtration chromatography on a Bio-Gel P-30 column (Legrand *et al.*, 1984). The iron-saturated *C*-peptic fragment of monoferric human lactotransferrin was prepared as previously described (Line *et al.*, 1976).

Isolation of the re-associated *N,C*-tryptic complex

The re-association of *N*- and *C*-tryptic fragments was obtained under iron-saturation conditions as follows: both fragments in solution in the acetic acid effluent from the Bio-Gel P-60 column were mixed in a ratio of 1:1 and, to the mixture thus obtained, a calculated amount of iron was added as an iron nitriloacetate reagent and the pH was then adjusted to 7.5 with 3 M-NaOH. The iron saturation was verified by determining the molar absorption at 465 nm ($\epsilon_{465} = 4750\ \mathrm{M}^{-1}\cdot\mathrm{cm}^{-1}$). The mixture was then twice dialysed for 24 h against a 0.1 M-NH_4HCO_3 solution and the *N,C*-tryptic complex was purified by gel-chromatography on Bio-Gel P-60 with 0.1 M-NH_4HCO_3 as eluent.

C.d. studies

Diferric lactotransferrin, monoferric *N*- and *C*-tryptic fragments, monoferric *C*-peptic fragment and diferric *N,C*-tryptic complex were dissolved in 0.214 M-NaF at a concentration of 0.1% and 5 ml portions were passed through a column (2 cm × 80 cm) of Bio-Gel P-30 equilibrated in 0.214 M-NaF. The proteins were eluted with the same solution and c.d. studies (Jobin-Yvon Dichrograph R. J. Mark III) were immediately performed in cylindrical cells with optical path lengths of 0.1 mm. For all computer calculations a mean amino acid residue M_r of 112 was used. The ellipticity was expressed as mean residual molar ellipticity $[\theta]$ in degrees·cm²·dmol⁻¹.

Prediction of secondary structure

For the prediction of α-helix and β-sheet regions, the conformational parameters of each amino acid residue as reported by Chou & Fasman (1974) and by Garnier *et al.* (1978) were used.

Other analytical techniques

Protein concentration was measured by the method of Lowry *et al.* (1951), with bovine serum albumin as standard. Estimation of M_r was performed by SDS/polyacrylamide-gel electrophoresis under reducing conditions (Kerckaert, 1978). Total iron content was determined by using a bathophenanthroline reagent kit (Biotrol, Paris, France).

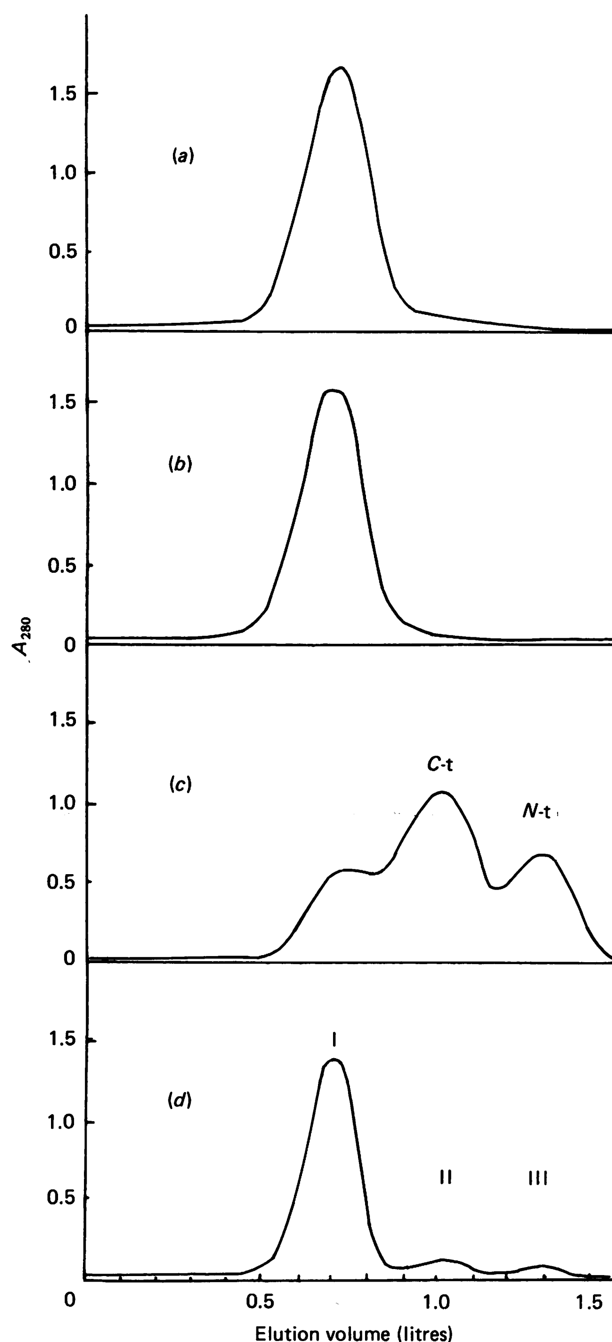

Fig. 1. Gel-filtration on Bio-Gel P-60

(*a*) Diferric lactotransferrin; (*b*) tryptic digest eluted with 0.1 M-NH_4HCO_3 with or without 4 M-urea or 1 M-KCl or ethanol (15%, v/v) or acetic acid (5%, v/v); (*c*) tryptic digest eluted with acetic acid (10%, v/v); (*d*) reconstituted *N,C*-tryptic complex of lactotransferrin eluted with 0.1 M-NH_4HCO_3. Fractions (5 ml) were collected at a flow rate of 8 ml/h. Abbreviations used: *N*-t and *C*-t, *N*- and *C*-terminal tryptic fragments.

RESULTS AND DISCUSSION

Characterization of the *N,C*-tryptic complex

The gel-filtration patterns of the tryptic digest of diferric lactotransferrin show that the separation of *N*- and *C*-tryptic fragments is only obtained when acetic acid is present at a concentration of over 5% (v/v) and probably as high as 10% (Figs. 1*b* and 1*c*). Under these conditions, iron is removed, as demonstrated by the disappearance of the absorbance at 465 nm. These results are in favour of the existence, between the two iron-saturated lobes of lactotransferrin, of non-covalent interactions that are stable at neutral pH and dissociable by acetic acid at concentrations of over 5% and probably as high as 10%. In addition, these interactions explain the presence of a single elution peak having the same chromatographic gel-filtration behaviour as the native lactotransferrin (Figs. 1*a* and 1*b*).

Re-association of the *N*- and *C*-tryptic fragments

The gel-filtration chromatography pattern on Bio-Gel P-60 (Fig. 1*d*) of the mixture of isolated *N*- and *C*-tryptic fragments (1:1 molar ratio) under non-denaturing conditions (0.1 M-NH_4HCO_3 as eluent), shows the presence, in addition to two minor peaks (peaks II and III), of a major component (peak I) having the behaviour of the native lactotransferrin (Fig. 1*d*).

SDS/polyacrylamide-gel electrophoresis of the three fractions confirms the existence of both *N*- and *C*-tryptic fragments in peak I and relates peaks II and III to the *C*- and *N*-tryptic fragments respectively. From the polyacrylamide-gel electrophoresis we can deduce that peak I is composed of an equimolar mixture of the *N*- and *C*-tryptic fragments (Fig. 2). These results suggest that the interaction between the *N*- and *C*-tryptic fragments is reversible. Moreover, from the quantity of *C*-tryptic (peak II) and *N*-tryptic (peak III) free fragments remaining after gel filtration, the yield of reassociation is estimated at 90%.

Restoration of the secondary structure by re-association of the *N*-and *C*-tryptic fragments

The interactions between the *N*- and *C*-tryptic fragments detected by gel-filtration chromatography were analysed by c.d. spectrometry. First, the α-helical content of human lactotransferrin and of its fragments was determined; then the modifications of the secondary structure induced by the re-association of the *N*- and *C*-tryptic fragments were detected from the difference c.d. spectra.

C.d. analysis of human lactotransferrin and of its tryptic fragments. The c.d. spectra of human lactotransferrin, and of *N*- and *C*-tryptic fragments under non-denaturing conditions from 190–250 nm are given in Fig. 3. As previously described by Mazurier *et al.* (1976), the far-ultraviolet spectrum of human lactotransferrin (Fig. 3, curve A) presents two negative bands of the same magnitude at 208 and 220 nm and an intense positive band at about 190 nm. The spectrum (Fig. 3, curve C) of the *C*-tryptic fragment (residues 283–703) resembles that of lactotransferrin, although the extremum at 208 nm appears now shifted at 210 nm and also slightly more intense. These effects, together with the virtual disappearance of the band at 220 nm, are probably due to a decrease in the α-helix content in the *C*-tryptic fragment compared to human lactotransferrin. In this hypothesis, however, a concomitant decrease of the positive band at 190 nm is expected as a result of the combination of the random and the α-helix spectra. The shape of the spectrum (Fig. 3, curve B) of the *N*-tryptic fragment (residues 3–281) in this respect illustrates perfectly the existence of a large amount of random structure besides helical and probably other regular structures as for instance β-sheets. From their circular-dichroism spectra, the number of amino acids involved in α-helix structure in lactotransferrin, in *N*-tryptic and in *C*-tryptic fragments has been calculated at 182, 31 and 94 respectively.

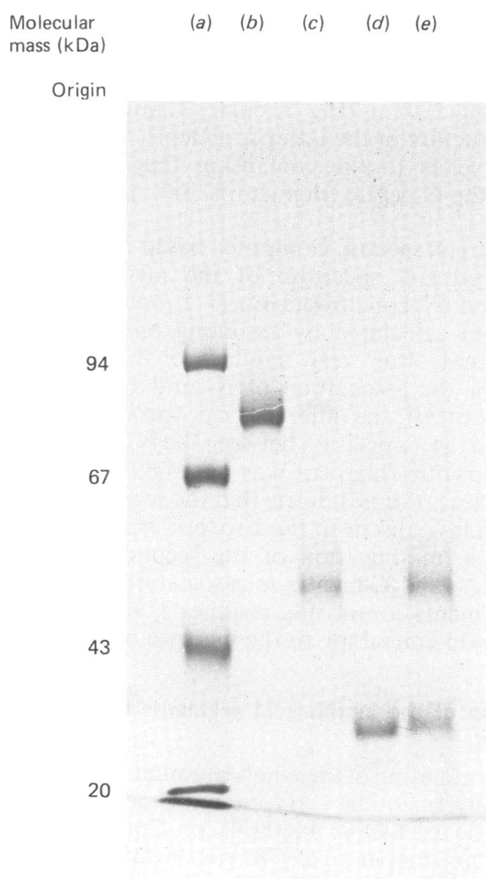

Fig. 2. SDS/polyacrylamide electrophoresis of tryptic fragments under reducing conditions

(*a*) Protein molecular-mass standards: phosphorylase *b* (94 kDa), albumin (67 kDa), ovalbumin (43 kDa), trypsin inhibitor (20 kDa); (*b*) native human lactotransferrin; (*c*) *C*-tryptic fragment; (*d*) *N*-tryptic fragment; (*e*) reconstituted *N*,*C*-tryptic complex (peak I of Fig. 1*d*).

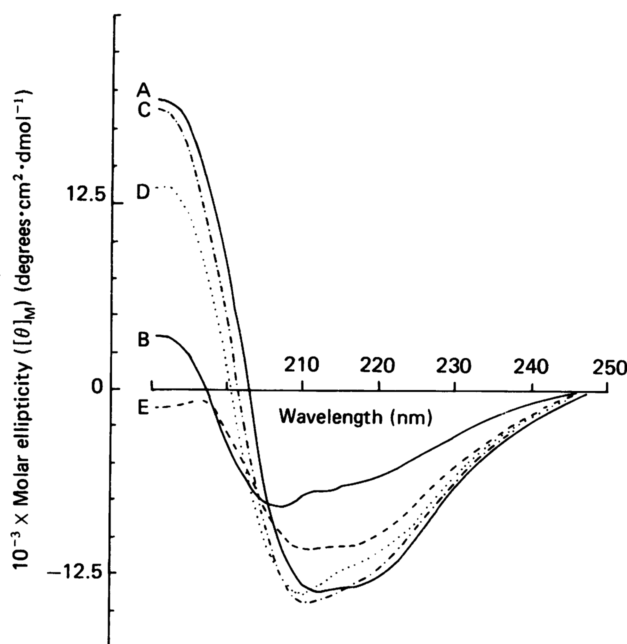

Fig. 3. C.d. spectra between 190 nm and 250 nm

A, Diferric lactotransferrin; B, *N*-tryptic fragment; C, *C*-tryptic fragment; D, *C*-peptic fragment; E, N2-glycopeptide.

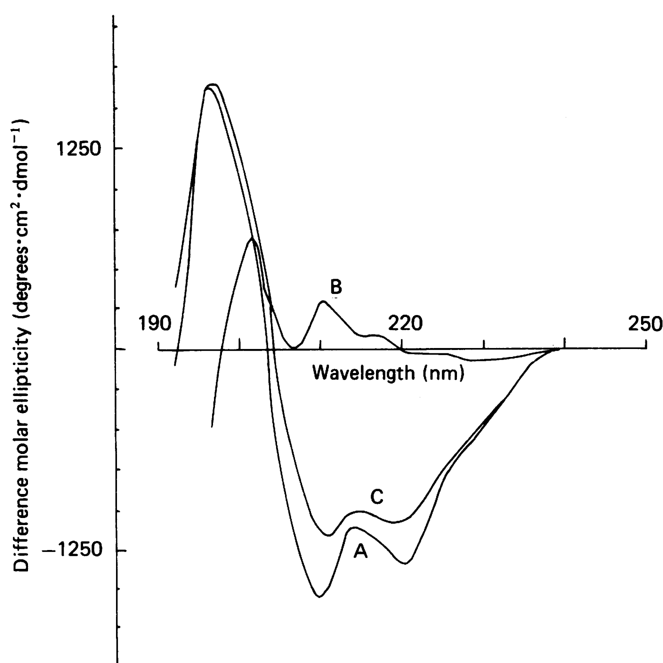

Fig. 4. Computer-calculated difference c.d. spectra

A, Difference between the measured spectrum of re-associated *N*- and *C*-tryptic fragments (1:1 molar ratio) and the sum of *N*- and *C*-tryptic-fragment spectra; B, difference between the mixture of N2-glycopeptide and *C*-tryptic fragments (1:1 molar ratio) and the sum of their respective spectra; C, difference between the mixture of *N*-tryptic and *C*-peptic fragments (1:1 molar ratio) and the sum of their spectra. The difference molar ellipticities refer to residues/mol.

Difference c.d. spectra. These were recorded between the *N*,*C*-tryptic complex (or the native diferric lactotransferrin) and the theoretical spectrum of the sum of the *N*- and *C*-tryptic fragments.

Computer-based comparison of the measured c.d. spectrum of the mixture of both *N*- and *C*-tryptic fragments (1:1 molar ratio), with the spectrum calculated by assuming one molecule of each fragment, is presented in Fig. 4 (curve A). The difference spectrum obtained shows two clearly distinct negative bands centred at 210 nm and 220 nm and a positive band centred at 196 nm. From these results, it is tempting to attribute the remaining ellipticities at 196 nm at 210 nm and at 220 nm to a gain in helical structure during the reassociation of the *N*,*C*-tryptic complex.

The differences based on the ellipticity at 222 nm indicate that a 14% increase in the α-helical content (17–18 amino acid residues) occurred during the re-association. The same experiment, involving a comparison of the spectrum of iron-saturated lactotransferrin and the measured spectrum of the mixture of both *N*- and *C*-tryptic fragments (results not shown) indicates that 88% of the α-helical content is restored in the re-associated *N*,*C*-tryptic complex. This result ties in well with those of the gel-filtration experiment.

Tentative localization of the segments involved in the interactions between the *N*- and *C*-terminal lobes of human lactotransferrin

In order to localize the segments of the *N*- and *C*-terminal lobes of lactotransferrin involved in the interactions between the *N*- and *C*-tryptic fragments, we analysed by c.d. (i) the association of the *C*-tryptic fragment with the N2-glycopeptide, which differs from the *N*-tryptic fragment by the absence of the peptide segments comprising residues 3–90 and residues 258–281, and (ii) the association of the *N*-tryptic fragment with the *C*-peptic fragment, which differs from the *C*-tryptic fragment by the lack of the peptide segment comprising residues 282–338.

C.d. analysis of N2-glycopeptide and *C*-peptic fragment. The first step was to compare the c.d. spectra of N2-glycopeptide and *C*-peptic fragment with *N*- and *C*-tryptic fragments previously described. The results given in Fig. 3 show that the shape of the spectrum of N2-glycopeptide presents, in contrast with that of the *N*-tryptic fragment, two extrema at 208 and 220 nm. This result indicates a higher percentage of α-helical structure in the N2-glycopeptide. In point of fact the number of amino acids in N2-glycopeptide involved in α-helical structure has been determined as 31. As this value is identical with the number of amino acids of the *N*-tryptic fragment involved in α-helical structure, it can be assumed that all the α-helical content of the *N*-tryptic fragment is located in the N2-glycopeptide (residues 91–357).

The comparison of the c.d. spectra of the *C*-tryptic and *C*-peptic fragments suggests, by the parallel decrease of the positive band at about 195 nm and of the negative band at about 220 nm (Fig. 3, curves C and D), a loss of α-helical structure in the latter fragments. The ellipticity at 222 nm leads to the conclusion that the α-helical content of the *C*-peptic fragment is 10% lower.

Difference c.d. spectra. Computer-based comparison of the measured c.d. spectrum of the mixture of both *N*-tryptic and *C*-peptic fragments (1:1 molar ratio), with the spectrum calculated by assuming one molecule of each fragment, was very similar to that previously obtained for the association of *N*- and *C*-tryptic fragments. In contrast, the difference c.d. spectrum obtained by analysing the association between the N2-glycopeptide and the *C*-tryptic fragment was not significant (Fig. 4, curve B). These results indicate that the segment (residues 282–338) that is absent in the *C*-peptic fragment has no effect on the modification of the secondary structure during the *N*- and *C*-tryptic re-association, whereas the peptide segments comprising residues 3–90 and residues 258–281 could contribute to the increase of the α-helical content.

Localization of the amino acid segments involved in the interactions

The determination of the α-helical content of lactotransferrin, N2-glycopeptide, *C*-tryptic and *C*-peptic fragments by using the predictive methods of Chou & Fasman (1974) and Garnier *et al.* (1978) gave results which tie in quite well with those obtained by c.d. (Table 1). Interestingly, the higher α-helical content of the *C*-tryptic fragment (residues 282–703) compared with that of the *C*-peptic fragment (residues 339–703) measured by c.d. was also detected by the two predictive schemes. In addition, careful examination of the secondary structure of the segment (residues 282–339) that is absent in the *C*-peptic fragment shows that only a part of this segment

Table 1. Number of amino acids participating in an α-helical conformation in peptide sequences of native lactotransferrin, tryptic and peptic fragments and re-associated *N*,*C*-tryptic complex

		No. of amino acids participating		
			Predictive	
Peptides	Amino acid sequences	Method . . . C.d.	Garnier *et al.* (1978)*	Chou & Fasman (1974)†
Lactotransferrin	1–703	182	162	160
Reconstituted *N*,*C*-tryptic complex	3–703	182	–	–
C-Tryptic fragment	282–703	94	106	85
C-Peptic fragment	339–703	85	94	77
N-Tryptic fragment	3–281	31	56	75
N_2-Glycopeptide	91–257	31	28	43

* α-Parameters > 75.
† $P(\alpha) > 1.05$.

Table 2. Conformational prediction of α-helix and β-sheet of the four segments involved in the interactions between the *N*- and *C*-tryptic fragments of human lactotransferrin

	Chou&Fasman (1974)		Garnier *et al.* (1978)	
Amino acid sequence	$P(\alpha)$	$P(\beta)$	α-Parameters	β-Parameters
19–24	1.03	1.11	134	24
45–50	1.08	1.23	60	156
50–58	1.17	0.94	138	9
264–276	1.15	0.99	164	−23.5

(residues 332–339) has a high probability of adopting an α-helical conformation. Consequently, the difference in helix content between the *C*-tryptic and *C*-peptic fragments could be attributed to this segment. On the other hand, the α-helical content of the *N*-tryptic fragment has been estimated at 20 and 27% by using the predictive methods of Garnier *et al.* (1978) and Chou & Fasman (1974) respectively, and at 11% by c.d. measurements. These results confirm that the dissociation of the non-covalent interactions between the two lobes of lactotransferrin leads to a decrease of the α-helical content of the polypeptide chain corresponding to the *N*-tryptic fragment (residues 3–281). Since the c.d. study has demonstrated that the segment corresponding to the N2-glycopeptide (residues 91–257) is not involved in these interactions, we have particularly examined the predictive secondary structure of segments between residues 3 and 90 and between 257 and 281 in order to locate more precisely the segments with high α-helix potential involved in the modifications of secondary structure described above. The results obtained are reported in Table 2. They show that peptide segments from residues 19 to 24, from 50 to 58 and from 264 to 276 exhibit a high probability of adopting an α-helical conformation, whereas the segment comprising residues 45–50 can adopt either an α-helical or a β-sheet structure. There are therefore strong grounds for thinking that these four segments are involved in the interactions between the *N*- and *C*-lobes of human lactotransferrin.

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