COMPARATIVE STUDY ON HISTIDINE MODIFICATION BY DIETHYLPYROCARBONATE IN HUMAN SEROTRANSFERRIN AND LACTOTRANSFERRIN

Milka A. KRYSTEVA*, Joël MAZURIER, Geneviève SPIK and Jean MONTREUIL

Laboratoire de Chimie Biologique, Université des Sciences et Techniques de Lille I et Laboratoire Associé au C.N.R.S. n° 217, B.P. n° 36, 59650 - Villeneuve d'Ascq, France

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1. Introduction

Serotransferrin (STF)** [1] and lactotransferrin (LTF) [2] are iron binding glycoproteins and the iron complex formed by LTF is known to be more stable especially at low pH [2]. It has generally been accepted that two or three tyrosine residues are involved in complex formation with each atom of Fe^{3+} in the molecule of STF [3–6] and LTF [7,8]. Electron paramagnetic resonance studies have shown that in STF two nitrogen atoms are also co-ordinated with each iron [6,9] and that, in the Cu^{2+} – LTF -bicarbonate complex, at least one nitrogen ligand is present at each metal binding site [8]. The results obtained by Bezkorovainy et al. [4,10] after alkylation with bromoacetic acid suggest that in STF the nitrogen atoms belong to histidine residues.

The present paper is dealing with identification of the nitrogen ligand present in the Fe^{3+} – LTF complex. The reactivity of His residues of both STF and LTF as well as that of their apoderivatives was studied using DEP. This specific reagent for His modification [11–13] reacts more rapidly (1.5 hr) than bromoacetic acid (12 days). Demonstration is brought about that (i) in LTF the nitrogen atoms coordinate with iron [8] belong to His residues; (ii) the reactivities of STF, LTF and of their apoderivatives toward the DEP reagent are quite different; (iii) modified His residues play a role in the color development in STF; (iv) carbethoxylation of apoSTF and apoLTF destroy only one iron binding-site.

2. Materials and methods

LTF was isolated from human milk [14] and its apoderivative was obtained according to Spik [15]. STF was prepared by the method of Roop and Putnam [16]. DEP was purchased from Sigma Chemical Co., USA.

For all calculations a mol. wt of 76 000 was assumed for LTF [14,15,17] and of 75 000 for STF [18]. The carbethoxylation was carried out according to Ovàdi et al. [11] using 0.1 M phosphate buffer, 0.1 M KCl at pH 6.0 and pH 7.4 at 14°C. The number of modified His residues was calculated assuming the molar absorption of 3.2×10^3 for carbethoxy-histidine [11] at pH 6.0 and 7.4 since no changes were detected in the molar absorption of the latter at both pH values. Difference spectra measurements were carried out by Beckman DB-6 recording spectrophotometer using 1 cm silica cells. Zeiss PMQ II was used too. Reduction of both proteins was performed according to Krysteva and Elödi [19]. The iron binding capacity of apoSTF

* Fellow of Ministère des Affaires étrangères. Present address : Department of Organic Chemistry, Institute of Chemical Technology, Sofia 56, Bulgaria.

***Abreviations* : DEP : diethylpyrocarbonate ; STF = iron saturated human serum transferrin ; LTF = iron saturated human lactotransferrin ; apoLTF = iron free lactotransferrin : apoSTF = iron free transferrin ; His = histidine : Tyr = tyrosine.

and apoLTF with modified His residues was determined according to Spik [15]. The estimation of iron content was performed by bathophenantroline modified method [15].

3. Results and discussion

3.1. *Reaction of STF, LTF and their apoderivatives with DEP*

Comparative titration of the four proteins by different amounts of DEP was carried out following the increase of difference spectra at 240 nm. It has been shown [15] that the iron binding site of STF undergoes alterations at pH 6.0, so the modification of STF was performed at pH 7.4. The modification of LTF was extended at pH 6.0 as well as at pH 7.4. Reaction of His in the four proteins studied was found to be time-dependent and complete in 1.5 h. The modification of all His residues, that is to say : 10 for LTF [15,17] and 17 for STF [14,20] was reached only with reduced and carboxy-methylated proteins in 8 M urea. The number of rapidly reacting His residues at pH 6.0 and 7.4 in iron-saturated and in iron-free STF and LTF are given in fig.1 and in table 1. The

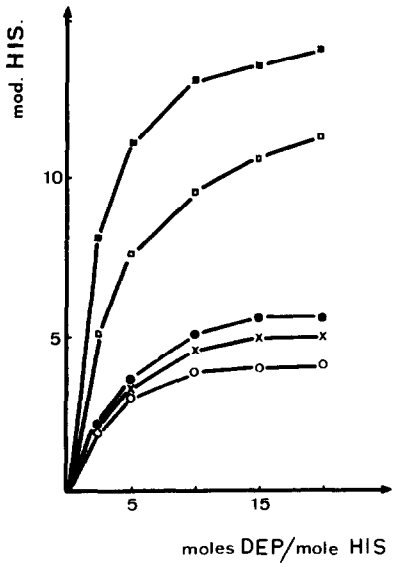

Fig.1. His modification dependence on DEP molarity. (-o——o-) LTF pH 6.0 ; (-x—x-) LTF pH 7.4 ; (-●——●-) apoLTF pH 6.0 and pH 7.4. ; (-□——□-) TF pH 7.4. ; (-■——■-) apoTF pH 6.0 and pH 7.4.

Table 1
His residues reactivity in STF and LTF toward diethylpyrocarbonate at different pH

Protein	Total His[a]	Rapidly reacting or exposed His residues	
		pH 6	pH 7.1
STF	17	14[b]	11
ApoSTF	17	14	14
LTF	10	4	5
ApoLTF	10	6	6

[a] Determined by amino acid analysis [14,17,20]
[b] In phosphate buffer at pH 6, STF loses its two iron atoms.

number of rapidly reacting His residues was also calculated using the method of Hegyi et al. [21].

The fact that 6 His residues of STF do not react with DEP at pH 7.4 can be explained if one considers that some His residues are involved in the iron binding sites and that some of them are deeply buried and unreactive toward DEP. At pH 6.0, in phosphate buffer, iron is completely removed from STF and the number of reacting His is identical to that found in apoSTF. So, it appears that at least 3 His residues are, if not buried, involved in the iron-binding sites. The reactivity of LTF toward DEP is quite different. Between pH 7.4 and 6.0 the conformational modification of LTF is less important, only one His residue appears to be exposed in iron-saturated protein. Iron is not removed because the solution remains colored. No important structural modification appears in apoLTF between pH 7.4 and 6.0. At least, if not buried, one His residue seems to be involved in the iron-binding site.

3.2. *Absorption changes due to the modification of His residues in iron saturated STF and LTF*

The reactions of His residues of STF with DEP causes the increase of the visible absorption intensity. The absorption increase depends on the extent of His modification. The absorption maximum at 465 nm is shifted by 4 nm to the longer wavelengths upon modification. About 28% increase of the STF initial absorption was found when 11 His residues were carbethoxylated. It could be assumed that new introduced carbethoxyl groups in exposed His residues are able to coordinate with iron. This phenomenon was not

Table 2
Iron binding capacity of His modified apoSTF and apoLTF

Protein	Number of modified His residues	Number of iron atoms per iron saturated transferrin molecule	Molar absorption of iron saturated protein at 465 nm
ApoSTF	0	2	4.10
	10	1	1.15
	14	1	1.90
ApoLTF	0	2	4.08
	3	1	1.60
	6	1	2.04

observed with LTF neither at pH 6.0 nor at pH 7.4. The absence of absorption changes at 465 nm upon modification of His residues in LTF leads us to assume that iron is not equally coordinatively saturated in the two proteins or that the number of His residues taking part in the complex formation is different in STF and LTF.

The difference spectra of modified proteins against native ones were recorded in 260–320 nm region. In the case of SRF a progressive increase of negative difference spectra with maxima at 287 and 292 nm as a function of His carbethoxylation was observed. Modification of 11 His residues of STF gave rise to -2800 M^{-1} cm^{-1} at 287 nm and -1800 M^{-1} cm^{-1} at 292 nm. These changes with LTF were negligible. The observed difference spectra could be attributed to some conformational changes resulting in the carbethoxylation of some His residues in STF since there was no absorption difference maximum at 270 nm indicating *O*-carbethoxylation of Tyr residues [22].

3.3. *Influence of His modification on the iron-binding properties of apoLTF and apoSTF*

In order to see whether the complete carbethoxylation of His residues is sufficient to prevent iron-binding, an iron resaturation was realized on His modified apoSTF and apoLTF. The level of iron specifically bound to the modified proteins was followed by the determination of the molar absorption at 465 nm and by the determination of the bound-iron by bathophenantrolin method. The results are summarized in table 2. The modification of 10 or 14 His residues in apoSTF resulted in the binding of only 1 atom iron and in the decrease of the STF molar absorption from 4.10×10^3 to 1.15×10^3 for 10 carbethoxylated His and to 1.95×10^3 for 14 carbethoxylated His. The modification of 3 or 6 His residues in apoLTF also resulted in the binding of only one iron atom and in the decrease of LTF molar absorption from 4.08×10^3 to 1.60×10^3 for 3 carbethoxylated His and to 2.04×10^3 for 6 carbethoxylated His. The results obtained show that the carbethoxylation of His residues of apoSTF and apoLTF causes the loss of only one of the iron binding sites.

4. Conclusion

These data demonstrate that, in STF and LTF molecules, His residues are directly involved in iron complex formation and that their participation in the iron binding sites is not identical in these two proteins. In the two proteins one of the iron-binding sites is deeply buried and unreactive toward DEP. The conformational changes of STF seems to be more pH dependent, in phosphate buffer, than that of LTF. The larger number of exposed and reactive His residues in STF as well as the influence of their modification on characteristic absorption at 465 nm refers to the difference in iron binding of the two proteins.

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