Biophysical Chemistry 24 (1986) 179–185
Elsevier

BPC 01068

INTERACTIONS OF MYOGLOBIN WITH UREA AND SOME ALKYLUREAS

II. CALORIMETRIC AND CIRCULAR DICHROIC STUDIES

Savo LAPANJE, Eva ŽEROVNIK and Zdenka KRANJC

Department of Chemistry and Chemical Technology, Edvard Kardelj University, Ljubljana, Yugoslavia

Received 24th July 1985
Accepted 1st March 1986

Key words: Myoglobin; Urea; Alkylurea; Calorimetry; Circular dichroism

The interactions of myoglobin with urea, methyl-, *N,N'*-dimethyl- and ethylurea were studied by means of calorimetry and circular dichroism (CD). The enthalpies of transfer from water to aqueous denaturant solutions are positive for the alkylureas and negative for urea. The difference is due to the presence of hydrophobic groups in the alkylureas. Gibbs free energies of transfer for urea solutions were obtained from preferential binding data determined previously. An attempt is made to interpret the values of the thermodynamic quantities in terms of various interactions between protein and denaturant. Analysis of the far-ultraviolet CD spectra reveals some differences in the denaturing activity of urea and the alkylureas, the latter being stronger denaturants than urea. Myoglobin displays relatively high stability towards these denaturants since concentrations above 5 M are needed for achieving major conformational changes.

1. Introduction

In the preceding publication [1] the solvation of myoglobin in aqueous urea and methyl-, *N,N'*-dimethyl- and ethylurea was dealt with. It has been found that the solvation of myoglobin in urea solutions is quite specific which is reflected in preferential hydration whereas the globular proteins studied thus far bind preferentially urea [2]. An attempt has been made to account for this peculiar behavior of myoglobin in terms of its high charge density. It has also been pointed out that for obtaining additional information about the interactions of myoglobin with these denaturants two methods, calorimetry and circular dichroism (CD), have been found to be very useful. The first gives the interaction energy, and the second information about conformational changes caused by the action of denaturants. On the basis of the data obtained by the two methods along with those ascertained by density measurements it is possible to arrive at important thermodynamic data characterizing the interaction between myoglobin and urea. For the alkylureas at high concentrations, i.e., above 4 M, no reliable data for preferential binding could be obtained so that only qualitative conclusions can be made. Analysis of the thermodynamic data for the interaction betwen myoglobin and ureas sheds light on the nature of this interaction for which in the literature quite divergent views can be found [3,4].

2. Experimental

The same myoglobin sample and reagents as for solvation studies were used [1]. Before preparing stock solutions, the protein was dialyzed against triply distilled water. The alkylureas were twice recrystallized from hot methanol.

Calorimetric experiments were performed with an LKB batch microcalorimeter. The initial protein concentrations were around 1.5% (w/v). The two compartments in the reaction cell were filled with (2.00 + Φ) ml protein solution and 4.00 ml urea (or alkylurea) solution, respectively, so that upon mixing a definite final concentration was obtained. Φ is the protein displacement volume, i.e., the product of protein mass times partial specific volume, 0.742 ml/g. The two compartments in the reference cell were filled with 2.00 ml water and 4.00 ml of the same urea (or alkylurea) solution. However, for final concentrations of urea (or alkylurea) above 4 M, the initial protein solutions were prepared in 4 and 6 M urea. For each protein concentration at least two experiments were performed. Since the two cells are not thermally balanced, their thermal response differing by about 3.5%, a separate blank experiment was made for each transfer. In the experiment the compartments in both cells were filled with 2.00 ml solvent and 4.00 ml denaturant solution. The apparent heat effect was corrected for in the real experiment.

CD spectra were recorded on a Roussel-Jouan mark III Dichrographe. In the experiments silica cells of 0.05 and 0.1 cm path length were used. The mean residue ellipticity $[\theta]_{\mathrm{mrw}}$ was calculated using the following equation

$$[\theta]_{\mathrm{mrw}} = \frac{M_0\theta}{100cl} \tag{1}$$

where M_0 is the mean residue molecular weight (116), θ the ellipticity, c the concentration (in g/ml) and l the pathlength (in dm).

3. Results and discussion

The values of the enthalpy of transfer of myoglobin from water to aqueous urea and alkylurea solutions are given in table 1. The enthalpies of transfer to urea solutions are seen to be negative throughout the concentration range, the absolute values increasing with increasing concentration of urea which is in accord with expectation. Namely, it is well known that the exothermic effect in the case of urea solutions is due to the fact that solvation changes involving the ionic groups on the surface of protein molecules represent the dominant contribution to the enthalpy of transfer [5]. The values range from hundreds of joules to several kilojoules per ionic group. The second contribution, the enthalpy of unfolding (i.e., the stabilization enthalpy; cf. below) is positive at 25°C, i.e., endothermic, and smaller [6]. In table 1 the values of the enthalpies of transfer of apomyoglobin are also included [7]. They are seen to be more negative than those of myoglobin reflecting undoubtedly the absence of the heme group. For proper comparison with other globular proteins it is appropriate to use the values for apomyoglobin.

Substitution of a hydrogen atom in the urea molecule with an alkyl group produces large changes in the interaction between myoglobin and the denaturant which is reflected in the enthalpies of transfer. They all become positive and increase

Table 1

Enthalpies of transfer of myoglobin to urea and alkylurea solution at 25°C

Final concentration (M)	ΔH_{tr} (kJ/mol) [a]				
		Urea	Methylurea	N,N'-Dimethylurea	Ethylurea
1.0	−60 ± 5	−67 ± 5 [b]		22 ± 2	
2.0	−110 ± 9	−145 ± 10	3 ± 1	125 ± 5	70 ± 5
3.0	−170 ± 11				
4.0	−215 ± 20	−300 ± 25	28 ± 2	370 ± 30	240 ± 20
6.0	−350 ± 30	−400 ± 30	67 ± 3	615 ± 50	450 ± 40
8.0	−505 ± 40	−590 ± 40			

[a] In all calculations, the molecular weight of myoglobin is taken to be 17800 and that of apomyoglobin 17200; uncertainties given are estimates.

[b] Values in this column are for apomyoglobin.

with increasing alkylurea concentration. Examination of table 1 further reveals that the enthalpies of transfer to solutions of *N,N′*-dimethylurea are largest; those to solutions of ethylurea are considerably smaller, and those to solution of methylurea smallest. In this case there is also a qualitative interpretation of the observed enthalpy values. The presence of nonpolar -CH_3 and -C_2H_5 groups in urea molecules gives rise to the hydrophobic interaction with similar, i.e., nonpolar, groups on the surface of protein molecules; the effect is positive [8,9]. The same groups also interact with protein peptide groups yielding another positive effect. Interactions with the ionic groups on the surface of protein molecules, on the other hand, are still negative but considerably smaller than in urea solutions (Lapanje, S. and Žerovnik, unpublished data). The overall balance of the various heat effects is thus positive and in agreement with the sequence of enthalpy values mentioned above.

As has been pointed out in the preceding paper [1], from preferential binding data the Gibbs free energy of transfer, ΔG_{tr}, can also be ascertained. For myoglobin in urea solutions enough data on preferential binding are available so that $\Delta G_{tr} = \mu_2^U - \mu_2^{H_2O}$, i.e., the difference in chemical potentials in urea solution and water, can be obtained by graphical integration of eq. 6 of the preceding paper. The values at 25°C are given in table 2. Also included are the corresponding enthalpy and entropy values. For reasons mentioned before, the enthalpy values are those for apomyoglobin. Owing to preferential binding of water, Gibbs free energy values are positive, whereas the enthalpy and entropy values are negative. In accordance with positive Gibbs free energy values, myoglobin undergoes very little conformational change up to 7 M urea (cf. fig. 1).

In table 2 the stabilization Gibbs free energy, ΔG_D^0, defined as Gibbs free energy necessary for converting the native protein to a randomly coiled one and the corresponding stabilization enthalpy, ΔH_D^0, and entropy, ΔS_D^0, at 25°C are also given. The value of ΔG_D^0, about 50 kJ/mol, is quite small which is a common phenomenon with globular proteins reflecting the fact that they are only marginally stable. What is unexpected, however, is the small value of ΔH_D^0, about 40 kJ/mol, that is due to the large temperature dependence of ΔH_D^0, i.e., the difference in heat capacity between the native and denatured state of myoglobin, $\Delta C_{P,D}$, 0.648 J/kg [6]. It has been claimed that at least 80% of the temperature dependence of the stabilization enthalpy can be accounted for by the negative heat of disruption of hydrophobic, i.e., nonpolar, contacts [6]. In other words, in myoglobin there are many such contacts which explains its high $\Delta C_{P,D}$ value. We will return to this topic when comparing myoglobin with β-lactoglobulin. Comparison of the values of ΔG_{tr} for urea solutions with that of ΔG_D^0 thus shows that the solvated form in these solutions is more stable than the native form in water, the additional Gibbs free energy being of the same order of magnitude as ΔG_D^0. The value of ΔG_{tr} to 8 M urea, for example, is 53 kJ/mol.

As mentioned in section 1, the negative prefer-

Table 2

Thermodynamic quantities (in kJ/mol) of transfer of myoglobin and β-lactoglobulin from water to aqueous urea solutions at 25°C

Concentration (M)	Myoglobin			β-Lactoglobulin [b]		
	$-\Delta G_{tr}$	$-\Delta H_{tr}$	$-T\Delta S_{tr}$	$-\Delta G_{tr}$	$-\Delta H_{tr}$	$-T\Delta S_{tr}$
4.0	−20 ± 10	300 ± 25	320 ± 35	50 ± 15	215 ± 40	165 ± 55
6.0	−40 ± 10	440 ± 30	480 ± 40	75 ± 15	440 ± 40	365 ± 55
8.0	−55 ± 10	590 ± 40	645 ± 50	100 ± 20	640 ± 40	560 ± 60
	(50) [c]	(40)	(−10)	(49) [c]	(188)	(139)

[a] ΔH_{tr} values are those for apomyoglobin (cf. table 1).

[b] Data from ref. 15.

[c] Values in parentheses are the stabilisation quantities, ΔG_D^0, ΔH_D^0 and $T\Delta S_D^0$, of myoglobin [8] and of β-lactoglobulin [14,16] at 25°.

ential binding of urea is an exception among the globular proteins studied thus far. Therefore comparison with one of those proteins that has been investigated very carefully, i.e., β-lactoglobulin, is indicated [10–14]. The values of thermodynamic quantities of stabilization and of transfer at 25°C are included in table 2. The value of $\Delta G_{\mathrm{D}}^{0}$ at 25°C for β-lactoglobulin is 50 kJ/mol, i.e., the same as that for myoglobin. However, the value of $\Delta H_{\mathrm{D}}^{0}$ is 188 kJ/mol and that of $T\Delta S_{\mathrm{D}}^{0}$ 139 kJ/mol; they are quite different from the corresponding values for myoglobin. It should also be noted that the values for β-lactoglobulin are only approximate.

The difference in $\Delta H_{\mathrm{D}}^{0}$ and $\Delta S_{\mathrm{D}}^{0}$ between myoglobin and β-lactoglobulin should reflect their different composition and structure. Myoglobin possesses about 79% α-helix [15] whereas β-lactoglobulin has 15% α-helix, 50% β-sheet and 15–20% β-turns [16]. Then there is the difference in the number of amino acid residues, 152 in myoglobin and 163 in β-lactoglobulin, which should not cause major concern. Assuming now, in accord with existing knowledge of factors responsible for folding, that the interpeptide hydrogen bonds and the hydrophobic interaction between nonpolar groups provide the main contribution to the enthalpy of folding [6], then the enthalpy of unfolding, i.e., $\Delta H_{\mathrm{D}}^{0}$, should contain two major contributions. The first involves the disruption of interpeptide hydrogen bonds and is thus positive, its value being somewhere 4 and 8 kJ per bond. The second contribution is the hydrophobic one; it is negative and quite smaller than the first, i.e., below 100 J per nonpolar contact [9]. However, there is a large number of such contacts in each protein molecule. The difference in $\Delta H_{\mathrm{D}}^{0}$ between myoglobin and β-lactoglobulin should thus largely be due to the difference in the number of nonpolar contacts. As has already been mentioned, in myoglobin there is a large number of such contacts which explains the high $\Delta C_{\mathrm{P,D}}$, 0.648 J/kg. Although for β-lactoglobulin no data on the number of nonpolar contacts are available, its $\Delta C_{\mathrm{P,D}}$ can be estimated at 0.4 J/kg [17,18]. This clearly shows that the hydrophobic interaction is smaller in β-lactoglobulin and explains the high positive value of $\Delta H_{\mathrm{D}}^{0}$. The values of $\Delta S_{\mathrm{D}}^{0}$ can be accounted for in a similar way. The contribution from disruption of interpeptide hydrogen bond is positive and that from disruption of nonpolar contacts negative, which again explains why the value of $\Delta S_{\mathrm{D}}^{0}$ for myoglobin is small and that for β-lactoglobulin quite large.

Owing to preferential binding of urea, the values of ΔG_{tr} for β-lactoglobulin are negative, and increase with increasing urea concentration (cf. table 2). The values of ΔH_{tr} are also negative and not very different from those for apomyoglobin. this is as expected since the interactions involved are the same. For the same reason, the values of ΔS_{tr} of myoglobin and β-lactoglobulin are also relatively close. Since for both proteins the values of ΔG_{tr} are rather small, this appears to be another instance of enthalpy-entropy compensation [19].

Since at high concentrations of the alkylureas preferential binding determinations were not feasible in most cases, ΔG_{tr} to alkylurea cannot be calculated. However, from the existing data (cf. table 2 of the preceding paper [1]) we infer that up to 4 M water is preferentially bound so that the values of ΔG_{tr} are positive.

Analysis of the CD data, on the other hand, gives invaluable information about the conformational changes and also allows one to answer questions about denaturing strength of urea and individual alkylureas. The CD spectra of myoglobin are presented in figs. 1 and 2. The interpretation of the spectra can be based on the comparison of the CD spectrum of the native protein in water with those in urea and alkylurea solutions. Therefore, the actual α-helix content in water is not of primary importance, but its change produced by individual denaturants. Examination of the CD spectra of myoglobin in urea solutions (fig. 1) reveals, in accordance with expectation, that with increasing urea concentration the values of $[\theta]$ decrease, i.e., they become less negative. However, up to 7 M the decrease is very small which means that the secondary structure has undergone little change. Then above 7 M large changes of $[\theta]$ occur reflecting unfolding of the native protein. The value of $[\theta]_{222}$ in 8 M urea is -8600 degree cm^{2} dmol^{-1} corresponding to about 20% of α-helical structure; in 9 M urea the value of $[\theta]_{222}$ drops to -5350 indicating that some helical structure is still preserved.

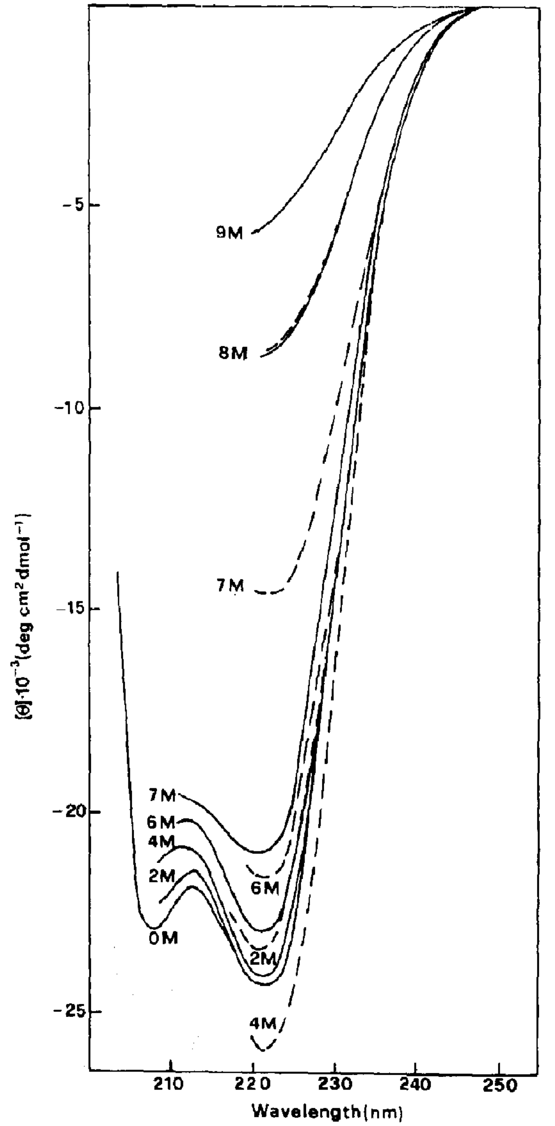

Fig. 1. Far-ultraviolet CD spectra of myoglobin in aqueous urea (———) and methylurea (— — —) solutions.

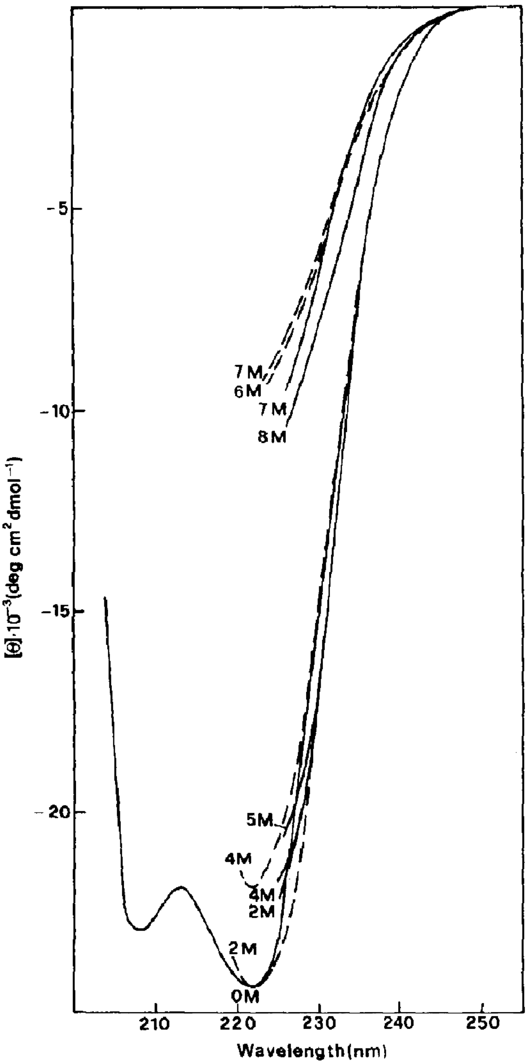

Fig. 2. Far-ultraviolet CD spectra of myoglobin in aqueous *N,N'*-dimethylurea (———) and ethylurea (— — —) solutions.

In methylurea solutions the CD spectra of myoglobin are clearly different from those observed in urea solutions. Thus, in 4 M methylurea the values of $[\theta]$ are more negative than those of native myoglobin. The peculiar behavior could be ascribed to partial structuring of the unordered regions [20]. In 6 M solution the values of $[\theta]$ are already distinctly less negative than those in 6 M urea. Between 6 and 8 M major conformational changes occur reflected in a large decrease of negative values of $[\theta]$. The values of $[\theta]_{222}$ in 8 M solution are about the same as in 8 M urea. As may be inferred from fig. 2, the denaturing activity of *N,N'*-dimethylurea at concentrations below

5 M is very low and not much different from that of urea. Large conformational changes occur above 5 M. The values of $[\theta]$ are relatively close to those in 8 M urea. However, between 7 and 8 M there is an inversion: in 8 M solution the values of $[\theta]$ are more negative indicating perhaps slight restructing. In ethylurea solutions the values of $[\theta]$ become less negative with increasing denaturant concentration. Major conformational changes occur between 4 and 6 M. The values of $[\theta]$ at 7 M are only slightly less negative than those in 6 M solution and close to those in 7 M N,N'-dimethylurea and 8 M urea. A feature characteristic of the action of urea and the alkylurea on myoglobin emerging from analysis of the CD spectra is its relatively high stability, since concentrations above 4 or 5 M are needed for bringing about major conformational changes.

In fig. 3 $[\theta]_{222}$ is plotted as a function of denaturant concentration. The denaturation midpoints, S_m, obtained from the plots, are given in the figure. This parameter which is a relative measure of the denaturing activity is defined as the concentration of denaturant needed to produce a 50% change in the quantity used to follow the denaturation process. As can be inferred from fig. 3, the denaturing activity increases with increasing hydrocarbon content and alkyl substitution of the amino group of urea. It should be noted that the values of S_m are in satisfactory agreement with those obtained previously from absorbance measurements at 409 nm and the mean residue rotation at 233 nm [21]. Owing to the strong solvent absorbance below 260 nm, the CD and rotation measurements could be less accurate than the spectrophotometric ones.

As has already been mentioned in the preceding paper [1], a quantitative thermodynamic analysis using binding and Setchenow constants allowed the calculation of S_m [21]. The calculated values seem to predict the correct trend of the denaturing activity of the ureas.

In conclusion, we may say that density and CD measurements as well as calorimetry have given valuable data on the interactions of myoglobin with urea and methyl-, N,N'-dimethyl- and ethylurea. The importance of preferential binding of the denaturant or water has been confirmed [14].

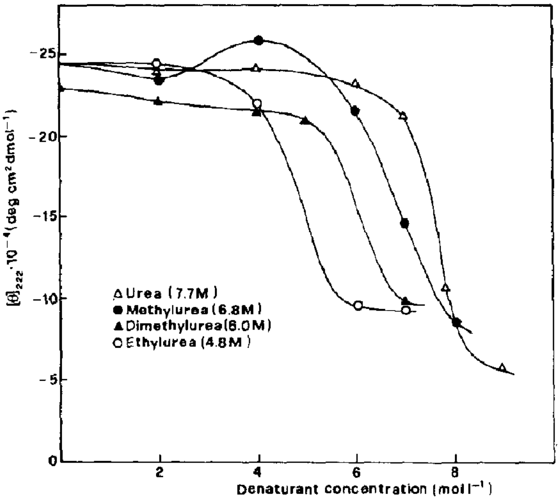

Fig. 3. The denaturation of myoglobin by various ureas followed by the mean residue rotation, $[\theta]$, at 222 nm. (△) urea, (●) methylurea, (▲) dimethylurea, (○) ethylurea. The values of the denaturation midpoint, S_m, are given in parentheses.

Preferential binding data were used in the calculation of Gibbs free energy of transfer of myoglobin from water to urea solutions. Since the corresponding enthalpies of transfer were obtained by calorimetry, the entropies of transfer could also be calculated. The values of thermodynamic quantities reflect various interactions between myoglobin and urea. For the alkylureas only the enthalpies of transfer were determined, and the hydrophobic interaction is seen to prevail over all the other interactions.

Acknowledgement

This study was supported by the Slovene Research Community.

References

1 E. Žerovnik and S. Lapanje, Biophys. Chem. 24 (1986) 53.
2 V. Prakash, C. Loucheux, S. Scheufele, M.J. Gorbunoff and S.N. Timasheff, Arch. Biochem. Biophys. 210 (1981) 455.
3 M. Roseman and W.P. Jencks, J. Am. Chem. Soc. 97 (1975) 631.

4 P.K. Nandi and D.R. Robinson, Biochemistry 23 (1984) 6661.

5 S. Lapanje, J. Škerjanc, S. Glavnik and S. Žibret, J. Chem. Thermodyn. 10 (1978) 425.

6 P.L. Privalov, Adv. Protein Chem. 33 (1979) 167.

7 S. Lapanje and E. Žerovnik, Vestn. Slov. Kem. Drug. 33 (1986) 35.

8 J.J. Savage and R.H. Wood, J. Solution Chem. 5 (1976) 733.

9 B.Y. Okamoto, R.H. Wood and P.T. Thomson, J. Chem. Soc. Faraday I 74 (1978) 1990.

10 J. Špan and S. Lapanje, Biochim. Biophys. Acta 295 (1973) 371.

11 J. Špan, S. Lenarčič and S. Lapanje, Biochim. Biophys. Acta 359 (1974) 311.

12 R.F. Greene, Jr and C.N. Pace, J. Biol. Chem. 249 (1974) 5388.

13 S. Lapanje, M. Lunder, V. Vlachy and J. Škerjanc, Biochim. Biophys. Acta 491 (1977) 482.

14 S. Lapanje, Physicochemical aspects of protein denaturation (Wiley Interscience, New York, 1978) ch. 4.

15 C.C. Chang, C.-S.C. Wu and J.T. Yang, Anal. Biochem. 91 (1978) 13.

16 L.K. Creamer, D.A.D. Parry and G.N. Malcolm, Arch. Biochem. Biophys. 227 (1983) 98.

17 J.N. DeWitt and G.M. Swinkels, Biochim. Biophys. Acta 624 (1980) 40.

18 N.C. Pace and C. Tanford, Biochemistry 7 (1968) 198.

19 R. Lumry and S. Rajender, Biopolymers 9 (1970) 1125.

20 S. Lapanje and Z. Kranjc, Biochim. Biophys. Acta 705 (1982) 111.

21 T.T. Herskovits, H. Jaillet and B. Gadekbeku, J. Biol. Chem. 245 (1970) 4544.