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Reversible cyclization of *S*-(2-oxo-2-carboxyethyl)-L-homocysteine to cystathionine ketimine

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Summary. S-(2-oxo-2-carboxyethyl)homocysteine (OCEHC), produced by the enzymatic monodeamination of cystathionine, is known to cyclize producing the seven membered ring of cystathionine ketimine (CK) which has been recognized as a cystathionine metabolite in mammals. Studies have been undertaken in order to find the best conditions of cyclization of synthetic OCEHC to CK and for the preparation of solid CK salt product. It has been found that ring closure takes place at alkaline pH and is highly accelerated in 0.5 M phosphate buffer. The sodium salt of CK has been prepared by controlled additions of NaOH to water-ethanol solution of OCEHC under N_2 atmosphere. A solid product is obtained which, dissolved in water, shows the spectral features of CK. Solutions of the sodium salt of CK show the presence of a pH depending reversible equilibrium with the open OCEHC form. Plot of the absorbance at 296 nm in function of pH indicates that at pH 9 the compound is completely cyclized while at pH 6 is totally in the open OCEHC form. At intermediate pHs variable ratios between the two forms occur. According to the results obtained by the spectral analysis, HPLC assays of the sodium salt of CK show different patterns depending on the pH of the elution buffer.

Keywords: Amino acids – Ketimine – Cystathionine

Abbreviations: CK, cystathionine ketimine; OCEHC, S-(2-oxo-2-carboxyethyl) homocysteine; HPLC, high performance liquid chromatography.

Introduction

Cystathionine ketimine (CK) is the product of the enzymatic (Ricci et al., 1983; Costa e al., 1986) and non enzymatic (Costa et al., 1984) monodeamination of L-cystathionine. The first product is the monoketo-monoamino acid which, in

alkaline conditions, spontaneously cyclizes to yield the ketimine. CK and its reduced product (cyclothionine) have been detected in mammals (Pecci et al., 1988a; Matarese et al., 1987) providing evidence on the biological significance of these products. CK has been prepared in two steps: first the solid form of S-(2-oxo-2-carboxyethyl)homocysteine (OCEHC) is obtained by reacting homocysteine with bromopyruvate (Ricci et al., 1983), in the second step a solution of OCEHC is left to cyclize at pH 8–8.5, monitoring the cyclization by the increase of the 296 nm absorbance (Ricci et al., 1983; Nardini et al., 1985). By this procedure an oxidation process can occur and the extent of cyclization is not known. In the present note we report the best conditions found for OCEHC cyclization. The preparation of solid samples of CK and the HPLC analysis of the product are also reported. The occurrence of variable ratios CK/OCEHC concentration at different pHs of the solution is described.

Materials and methods

DL- and L-homocysteine thiolactone hydrochloride and bromopyruvic acid were purchased from Sigma. Spectra were recorded with a Varian DMS 90 spectrophotometer. HPLC analyses were carried out with a Waters chromatograph equipped with two Model 501 pumps, a Model 680 gradient controller and a U6K sample injector. Eluates from the column were detected by a Waters Model 490 variable wavelength detector. The column was a 250 mm × 4 mm Hypersil ODS, 5 micron, equilibrated and eluted with 50 mM potassium phosphate buffer at various pHs. Flow rate: 1 ml/min at room temperature.

Results

Preparation of OCEHC

OCEHC has been prepared as reported earlier (Ricci et al., 1983) with minor modifications. In brief: 154 mg (1 mmol) of DL or L-homocysteine thiolactone hydrochloride are dissolved in 2 ml 1 *N* NaOH and heated, under N_2 bubbling, in the boiling water bath for 3 min to open the thiolactone. The solution cooled to room temperature under N_2 is added with a solution of 0.2 g bromopyruvic acid in 5 ml water. After 5 min the reaction is complete (nitroprusside test) and the solution is passed through a 1 × 5 cm column of Dowex 50 × 8 (H^+ form, 200–400 mesh) which is eluted with water. Fractions between 70 and 120 ml (iodoplatinate test) are collected and dryed with a rotary evaporator at 40°C to obtain about 70 mg of product (analytical data are those reported in Ricci et al., 1983).

Cyclization to CK

A solution 0.5 mM of OCEHC in water (resulting pH = 3.5) shows the spectral profile (a) reported in Fig. 1A with a maximum at 265 nm ($\varepsilon = 800\ M^{-1}\ cm^{-1}$). The same solution rised and mantained at pH 8 by controlled addition of 2 *N* NaOH shows a slow increase of absorbance at 296 nm typical for the formation of the seven membered ketimine ring (Ricci et al., 1983). We have now found that phosphate buffer exhibits a strong effect on the cyclization rate. As seen in

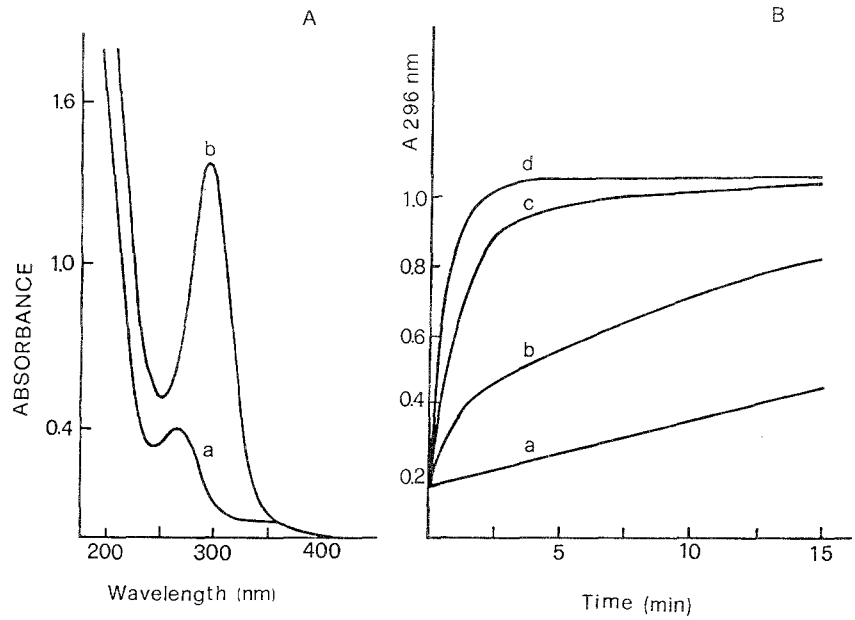

Fig. 1. A Absorption spectra of OCEHC 0.5 mM in H_2O (*a*) and in 0.5 M K-phosphate buffer, pH 8 (*b*). **B** Increase of absorbance at 296 nm with time of solutions 0.5 mM of OCEHC in H_2O adjusted at pH 8 with 2 N NaOH (*a*) and in K-phosphate buffer, pH 8, 0.01 M (*b*), 0.1 M (*c*) and 0.5 M (*d*)

Fig. 1B, ring closure at pH 8 depends on the ionic strength of the buffer being almost immediate at concentration of phosphate 0.5 M. The solution of OCEHC in 0.5 M phosphate buffer, pH 8, shows the spectral profile (b) of Fig. 1A with the maximum at 296 nm and minimum at 250 nm. The absorbance slowly decreases if the solution is not preserved from the contact with air.

Dissolved in absolute ethanol or methanol, OCEHC gives the curve a in Fig. 2 with the maximum at 315 nm ($\varepsilon = 5000\ M^{-1}\ cm^{-1}$). Elimination of the solvent by distillation in vacuum followed by dissolution of the residue in water regenerates the open OCEHC form with the spectrum identical to that of Fig. 1A. In Fig. 2 the spectra of OCEHC dissolved in 1 *N* NaOH (curve b) and in 1 *N* HCl (curve c) are also reported.

Preparation of the CK sodium salt

Solid CK has been prepared in the form of sodium salt as follows: 22 mg OCEHC (0.1 mmol) are dissolved in 1 ml water, previously boiled and cooled to room temperature under N_2 bubbling. 6 ml of 96° ethanol are added and, under gentle N_2 bubbling, 4 portions of 25 μl 2 *N* NaOH are added over a period of 20 min. The solution is left to complete the cyclization (about 15 min) then dryed in the rotovap at room temperature. The residue is dissolved in 0.25 ml water and added with 5 ml absolute ethanol. The precipitate is removed by centrifugation and the supernatant is dryed in the rotovap at room temperature to obtain about 10 mg of white powder. When dissolved in H_2O (at concentration 0.5 mM the resulting pH was 9.3) the product gives the spectrum with the absorbance

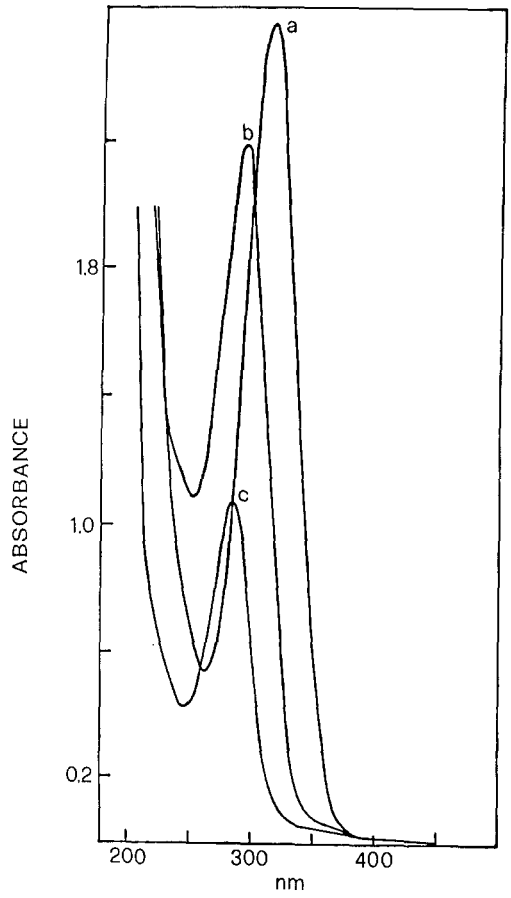

Fig. 2. Spectral curves of 0.5 mM OCEHC in absolute ethanol (*a*); 0.1 N NaOH (*b*) and 0.1 N HCl (*c*)

at 296 nm typical of the ketimine ring. Equal molar solutions of OCEHC (MW = 221.2) and of CK salt, assumed to be in the disodium form (MW = 247.2), exhibit the same absorbance at 296 nm in 100 mM phosphate buffer, pH 9.2. Solid CK salt, stored in the dessiccator under vacuum, is stable for at least one month.

Evidence of the equilibrium between OCEHC and CK by UV changes

The change of absorbance at 296 nm obtained by changing pH of the CK salt solution is seen in Fig. 3A. For this experiment solutions 0.5 mM of the CK salt in 100 mM phosphate buffer pH 7.4 were brought to higher or lower pHs by addition of controlled amounts of 6 *N* NaOH or 6 *N* HCl respectively. At the indicated values of pHs, changes of the absorbance at 296 nm were followed until constant values were obtained (about 5 min) then spectra were registered and the absorbances at 296 nm reported in the diagram. At pH values between 5 and 6 the spectra were identical with that reported above for the OCEHC form (Fig. 1A), indicating the complete opening of CK. Increasing the pH, the absorbance at 296 nm increases and reaches the maximum value at pH 9 (ε =

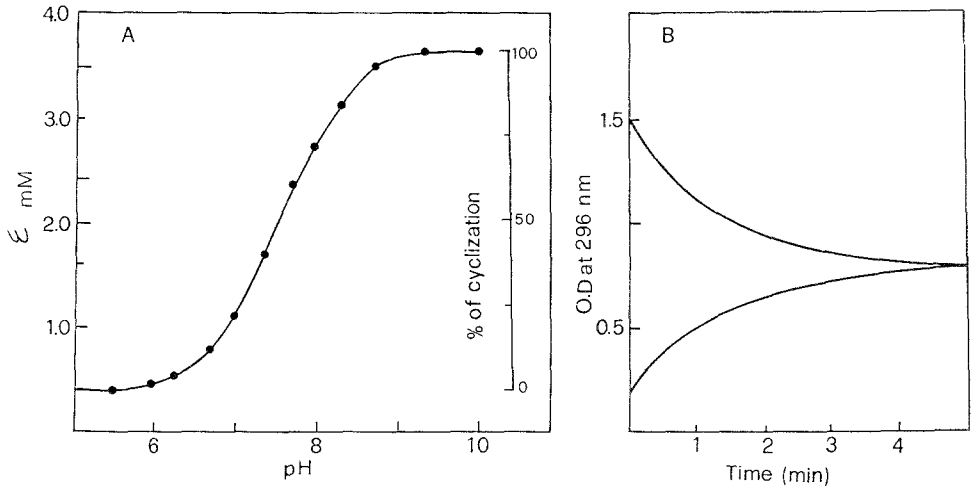

Fig. 3. A Molar absorbance at 296 nm and related CK/OCEHC ratio (expressed as % of cyclization) in function of pH. Experimental details in the text. **B** Absorbances changes at 296 nm with time of solutions 0.5 mM CK sodium salt and OCEHC in 0.5 M K-phosphate buffer, pH 7.4

3600 M^{-1} cm^{-1}) indicating that in these conditions the compound is completely cyclized. At intermediate pHs different CK/OCEHC ratios exist which can be calculated from the values of the absorbance at 296 nm. The effect of the pH in the equilibrium of the CK-OCEHC couple is operative from pH 6 to 9. Changes of UV absorbance below (in 1 *N* HCl) and above (in 1 *N* NaOH) this range should be imputable to structural modification of different nature.

The changes of absorbance at 296 nm in function of time when either the open OCEHC or the cyclic CK salt are dissolved in 0.5 M phosphate buffer, pH 7.4 is seen in Fig. 3B. As expected, after the equilibrium is reached (about 5 min), the two solutions exhibit the same value of absorbance at 296 nm *i.e.* the same ratio OCEHC/CK. From the data reported in Fig. 3 it is possible to calculate that at the physiological pH 7.4 the compound is 40% in the cyclic CK form.

HPLC analysis

CK has been determined, so far, by using gas liquid chromatography after diazomethane derivatization (Matarese et al., 1984; Ricci et al., 1990), by HPLC after derivatization with the Edman reagent (Pecci et al., 1988b; Antonucci et al., 1990) and more recently by derivatization with 2,4-dinitrophenylhydrazine (work in progress). The amino acid analyzer has been also used for this assay (Cavallini et al., 1985a; Costa et al., 1985). While the first two procedures allow determination of the compound in the cyclized form, the last two procedures are done in acidic conditions known to open CK. We have now found that direct HPLC analysis of the compound permits to distinguish between the open and the cyclized form. CK and OCEHC are eluted close to the elution front and close to each other, with OCEHC emerging first. Elution profiles, monitored at

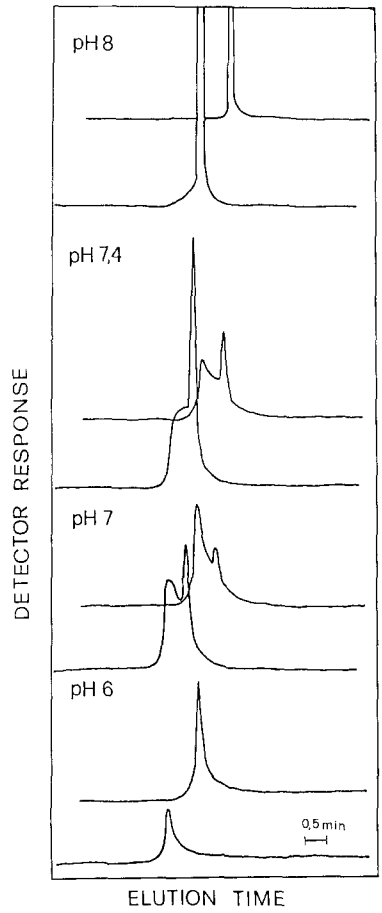

Fig. 4. Reverse-phase HPLC of CK sodium salt (5 nmol) eluted with 50 mM K-phosphate buffer at various pH. Detection at 296 nm (lower chromatogram) and 265 nm (upper chromatogram). Experimental conditions as reported in Materials and methods

296 nm and at 265 nm, of CK salt dissolved and eluted with 50 mM phosphate buffer at different pHs are illustrated in Fig. 4. The results agree with those reported above indicating that while at pH 6 the compound exists in the open form at pH 8 prevails the cyclyzed form. At intermediate pHs the presence of the equilibrium between the two forms is clearly evidenced.

Discussion

Compared with other ketimines, the ring closure of OCEHC to CK is a slower process and requires higher pH and ionic strength to be complete. At physiological pH 7.4 CK is in reversible equilibrium with the open form with CK accounting for 40% of the total. This conclusion is in accord with the reported presence in vivo of both the products of the enzymatic reduction of CK and of OCEHC, *i.e.* cyclothionine (Matarese et al., 1987; Cavallini et al., 1985b) and S-(2-hydroxy-2-carboxyethyl)homocysteine (Watanabe et al., 1991).

Sulfur containing cyclic ketimines are known to be present in large part in the enimine form (Ricci et al., 1982). In the case of CK the compound should be

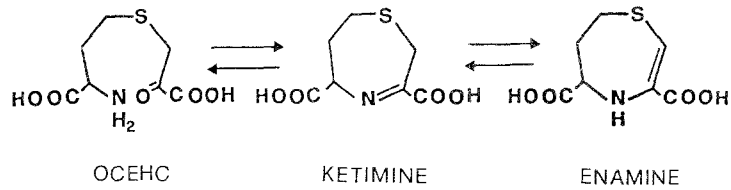

Fig. 5. Equilibrium scheme between the open OCEHC and cyclized CK forms

therefore present in the forms indicated in Fig. 5 with the equilibrium shifted towards the right side in alkaline conditions.

The seven membered ring of CK is asimmetric with respect to sulfur atom and two isomers are expected to occur. The present note has been limited to the study of the isomer with the double bond in the shorter carbon chain moiety of the ring, which is the preferred product of the enzymatic deamination of cystathionine. The isomer with the double bond on the other side of the ring has been prepared earlier enzymically (Ricci et al., 1983) but not further characterized.

As conclusion of this investigation it appears that the simplest way to prepare CK in solution is to dissolve OCEHC in 100 mM phosphate buffer pH 9 under N_2 and dilute the solution to the desired concentration of buffer and CK. When the presence of the buffer is undesirable the preparation of the CK salt as described in this note is the choice. In any case the content of cyclized CK in the solution at the pH used has to be established by spectral analysis using the molar extinction given above.

Acknowledgements

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