Isolation of S-[2-carboxy-1-(1*H*-imidazol-4-yl)ethyl]-3-thiolactic acid, a new metabolite of histidine, from normal human urine and its formation from S-[2-carboxy-1-(1*H*-imidazol-4-yl)ethyl]cysteine

Masahiro KINUTA,* Toshihiko UBUKA, Wen-Bin YAO, Yuan-Qing ZHAO and Hiroo SHIMIZU

Department of Biochemistry, Okayama University Medical School, Okayama 700, Japan

S-[2-Carboxy-1-(1*H*-imidazol-4-yl)ethyl]-3-thiolactic acid (CIE-TL), a novel imidazole compound with a sulphur-containing side chain, was isolated from normal human urine by ion-exchange column chromatography, and characterized by physicochemical analyses involving m.s., i.r. spectrophotometry, high-voltage paper electrophoresis and elemental analysis as well as chemical synthesis. CIE-TL was synthesized by the reaction of S-[2-carboxy-1-(1*H*-imidazol-4-yl)ethyl]cysteine (CIE-Cys) with NaNO₂ in HCl. CIE-TL was also formed during enzymic degradation of CIE-Cys by rat liver or kidney homogenate in a phosphate buffer, possibly via the metabolic intermediate S-[2-

carboxy-1-(1*H*-imidazol-4-yl)ethyl]-3-thiopyruvic acid, and this was accompanied by the formation of 3-[(carboxymethyl)thio]-3-(1*H*-imidazol-4-yl)propanoic acid, a compound previously found in human urine [Kinuta, Yao, Masuoka, Ohta, Teraoka and Ubuka (1991) *Biochem. J.* **275**, 617–621]. These results suggest that CIE-Cys [Kinuta, Ubuka, Yao, Futani, Fujiwara and Kurozumi (1992) *Biochem. J.* **283**, 39–40] is a physiological precursor of the urinary compounds and that L-histidine is metabolized in part via an alternative pathway initiated by the adduction of natural thiol compounds such as cysteine and GSH to urocanic acid, the first catabolite of histidine.

INTRODUCTION

In the first step of histidine catabolism, urocanic acid is formed by the action of histidine ammonia-lyase (EC 4.3.1.3), which is usually only found in liver and epidermis (Zannoni and La Du, 1963). In liver, urocanic acid is metabolized by urocanate hydratase (EC 4.2.1.49) to 4,5-dihydro-4-oxo-5-imidazolepropanoic acid, and this compound is further catabolized ultimately to CO₂ and water. In the epidermis, however, histidine metabolism terminates in the formation of urocanic acid, as urocanase is not present in the skin (Barden and Pathak, 1967; Scott, 1981). Therefore urocanic acid accumulates in the epidermis, although its content changes in response to exposure of the skin to u.v. light (Hais and Štrych, 1968; Kral et al., 1968; Wadia et al., 1975). On the other hand, two novel imidazole compounds with sulphur-containing side chains have been isolated from normal human urine and these were determined to be S-[2-carboxy-1-(1*H*-imidazol-4-yl)ethyl]-2-thioacetic acid (CIE-TA) {3-[(carboxymethyl)thio]-3-(1*H*-imidazol-4-yl)propanoic acid} (Kinuta et al., 1991) and S-[2-carboxy-1-(1*H*-imidazol-4-yl)ethyl]cysteine (CIE-Cys) (Kinuta et al., 1992a,b). These findings suggest the participation of natural thiol compounds such as L-cysteine and GSH in the metabolism of urocanic acid. In order to clarify the origin of the urinary compounds, S-[2-carboxy-1-(1*H*-imidazol-4-yl)ethyl]glutathione (CIE-SG), which is an analogue of urocanic acid adducted with GSH, was synthesized (Kinuta et al., 1991, 1993). Enzymic degradation of this compound by rat kidney homogenate produced CIE-Cys via a metabolic intermediate, N-{S-[2-carboxy-1-(1*H*-imidazol-4-yl)ethyl]cysteinyl} glycine (CIE-Cys-Gly), as described previously (Kinuta et al., 1993). CIE-TA has also been demonstrated to be a product of enzymic degradation of CIE-Cys in rat liver (Kinuta et al., 1991) or kidney homogenate (Kinuta et al., 1993) in phosphate buffer.

In further studies on CIE-Cys metabolism, an unidentified urinary compound was found on a high-voltage paper electrophoretogram sprayed with Pauly's reagent. This compound has now been determined to be S-[2-carboxy-1-(1*H*-imidazol-4-yl)ethyl]-3-thiolactic acid (CIE-TL) {3-[(2-hydroxy-2-carboxyethyl)thio]-3-(1*H*-imidazol-4-yl)propanoic acid}. The present paper describes the isolation, determination of the structure and the proposed precursor of this urinary compound. A novel alternative pathway of L-histidine metabolism which produces CIE-TL is also discussed. Figure 1 shows the structural formulae of CIE-TL and its proposed precursor S-[2-carboxy-1-(1*H*-imidazol-4-yl)ethyl]-3-thiopyruvic acid (CIE-TP) {3-[(2-oxo-2-carboxyethyl)thio]-3-(1*H*-imidazol-4-yl)propanoic acid}.

MATERIALS AND METHODS

Chemicals

Ion-exchangers and chemicals used in the present work were as described previously (Kinuta et al., 1991, 1993). CIE-Cys and CIE-TA were synthesized by the addition of L-cysteine and 2-mercaptoacetic acid respectively to *trans*-urocanic acid [*trans*-3-(1*H*-imidazol-4-yl)acrylic acid] as described previously (Kinuta et al., 1991, 1992a). Pauly's reagent (Pauly, 1904; Macpherson, 1946), chloroplatinate reagent (Toennis and Kolb, 1951) and ninhydrin (triketohydrindene hydrate) reagent (Kinuta et al., 1993) were used as described previously (Kinuta et al., 1991).

Spectroscopy and paper electrophoresis

Fast-atom-bombardment m.s. (f.a.b.-m.s.) and chemical-ionization m.s. (c.i.-m.s.) with a direct-inlet system were carried out on a Shimadzu 9020-DF gas chromatograph-mass spectrometer equipped with a Shimadzu SCAP 1123 data system and a 7240A plotter printer (Hewlett-Packard) as described pre-

Abbreviations used: CIE, S-[2-carboxy-1-(1*H*-imidazol-4-yl)ethyl]-; TL, 3-thiolactic acid; TA, 2-thioacetic acid; TP, 3-thiopyruvic acid; TAal, 2-thioacetaldehyde.

* To whom correspondence should be sent.

Figure 1 Structural formulae of imidazole compounds

CIE-TL, *S*-[2-carboxy-1-(1*H*-imidazol-4-yl)ethyl]-3-thiolactic acid: $R = -S-CH_2-CH(OH)-CO_2H$; CIE-TP, *S*-[2-carboxy-1-(1*H*-imidazol-4-yl)ethyl]-3-thiopyruvic acid: $R = -S-CH_2-CO-CO_2H$.

viously (Kinuta et al., 1992a). I.r. spectra (KBr pellet) were recorded with a Japan Spectroscopic A-120 i.r. spectrophotometer. High-voltage paper electrophoresis was performed on Whatman 1Chr paper (Whatman, Maidstone, Kent, U.K.) in a buffer (pH 3.1) consisting of pyridine/acetic acid/water (1:20:179, by vol.) (Ubuka, 1962) at a potential gradient of 100 V/cm for 45 min. Relative mobilities of compounds on the paper electrophoretogram, termed the mR_{TLA} value, were determined as described previously (Kinuta et al., 1991).

Enzymic degradation

Male Wistar rat liver and kidney homogenates were prepared as described previously (Kinuta et al., 1991, 1993). Enzymic degradation of CIE-Cys was carried out by incubating 8 ml of a reaction mixture containing 3 ml of the homogenate and 1 mmol of CIE-Cys in 0.1 M potassium phosphate buffer, pH 7.4, containing 5 mM EDTA at 37 °C for 2 h. The reaction was terminated by the addition of 2 ml of 50% (w/v) trichloroacetic acid, and the reaction mixture was centrifuged at 1800 *g* for 30 min to obtain the supernatant. The supernatant was then desalted on a Dowex 50 column (H^+ form, 2 cm $\times$ 20 cm) by a previous method (Kinuta et al., 1993). Reaction mixtures without the substrate (CIE-Cys) or with homogenate preheated at 100 °C for 3 min instead of the above homogenate were used as controls. A smaller-scale reaction of CIE-Cys (25 μ mol) with 25% (w/v) rat kidney homogenate in phosphate buffer, pH 7.4, was carried out as previously described (Kinuta et al., 1993).

Measurement of CIE-TL

Quantitative analysis of CIE-TL was carried out by recording the absorbance at 500 nm after reaction with diazotized sulphanilic acid in a similar method to that used for CIE-TA and CIE-Cys described previously (Kinuta et al., 1993). Values obtained were linear between 3 and 50 μ M CIE-TL. A blank was prepared in the same manner omitting the imidazole compound only.

RESULTS

Chemical synthesis of CIE-TL

The compound CIE-TL was made by diazotization of CIE-Cys with $NaNO_2$ in HCl as follows: 2 ml of 2 M HCl was added slowly to a 2 ml solution of CIE-Cys (1 mmol) in 1.25 M $NaNO_2$, below 4 °C with stirring; the reaction mixture was incubated for 70 min below 4 °C and then for 7 h at 16–18 °C. When the reaction was finished, the mixture was adjusted to pH 4.0 with 2 M NaOH and filtered. The filtrate was desalted on a Dowex 50 column (1.5 cm $\times$ 6 cm) and chromatographed on a Dowex 1 column (1.5 cm $\times$ 13.5 cm) with the successive eluents (240 ml of

each) 0.02 M, 0.05 M, 0.1 M and 0.2 M acetic acid, 30 ml portions being collected. As checked by high-voltage paper electrophoresis, the major product was eluted between 0 and 60 ml of the 0.2 M acetic acid, and crystals with little contamination were obtained from the eluates. The crystals were recrystallized twice from ethanol/water to give 119 mg (yield 45.8%) of CIE-TL. The purity of the crystals was determined by paper electrophoresis. The compound gave a reddish band at mR_{TLA} 0.38 on the paper electrophoretogram sprayed with Pauly's reagent. This compound gave a positive reaction with the chloroplatinate reagent, but the ninhydrin test was negative. CIE-TL resisted treatment with 6 M HCl at 100 °C for 2 h (analytical data: calc. for $C_9H_{12}N_2O_5S$: C, 41.53; H, 4.65; N, 10.76%; found: C, 41.72; H, 4.71; N, 10.58%). Signals on the f.a.b.-mass spectrum (Figure 2a) of the synthesized compound were assigned as follows: m/z 261 [MH^+ (the molecular ion plus proton)], 140 [$MH^+ - [S-CH_2-CH(OH)-CO_2H]$], and 95 (140 - CO_2H). In the c.i.-mass spectrum, a signal of MH^+ at m/z 261 was small (2.4%); instead, signals of $MH^+ - H_2O$, $MH^+ - CO_2$, $MH^+ - [HS-CH_2-CH(OH)-CO_2H]$ and $[C_3H_3N_2-CH=CH_2]H^+$ were given at m/z 243 (7.6%), 217 (15.1%), 139 (base) and 95 (81.2%) respectively.

Isolation of CIE-TL from human urine

As checked by high-voltage paper electrophoresis, the desired compound with mR_{TLA} 0.38 was purified on successive ion-exchange columns, Diaion SK no. 1, Diaion SA no. 100, Dowex 50 and Dowex 1 essentially by the method (Kinuta et al., 1991) used previously for isolation of CIE-TA. Further purification was carried out on a Dowex 1 column (1.3 cm $\times$ 20 cm) eluted

Figure 2 F.a.b.-mass spectra

(a) Synthesized CIE-TL; (b) the isolated urinary compound; (c) the compound formed by the enzymic degradation of CIE-Cys.

with 0.2 M acetic acid. Crystals were obtained from the 60–150 ml fractions of the eluates, and recrystallized from ethanol/water to give approx. 9 mg of crystals from a total of 120 litres of healthy human urine. Analytical data found: C, 41.79; H, 4.68; N, 10.62%. These data indicated a molecular formula of $C_9H_{12}N_2O_5S$ (M_r , 260). This compound gave positive reactions with Pauly's reagent and the chloroplatinate reagent, but the ninhydrin test was negative. The f.a.b.-mass spectrum (Figure 2b) and the i.r. spectrum of the urinary compound were identical with those of CIE-TL synthesized as described above. From these results, the isolated urinary compound was determined to be CIE-TL.

Formation of CIE-TL by enzymic degradation of CIE-Cys

Enzymic degradation of CIE-Cys (1 mmol) was performed by incubation with rat liver or kidney homogenate in phosphate buffer, pH 7.4, at 37 °C. The desalted products were further chromatographed on a Dowex 1 column (acetate form, 1.5 cm × 15 cm) with the successive eluents (260 ml of each) water, 0.02 M, 0.05 M, 0.1 M, 0.2 M, 0.5 M and 2 M acetic acid, with 20 ml portions being collected. As checked by paper electrophoresis, the desired compound with mR_{TLA} 0.38 was eluted between 40 and 120 ml of the 0.2 M acetic acid, and crystals with little contamination were obtained from the eluates. The crystals were recrystallized from ethanol/water to give 37.2 mg (yield 14.3%) and 23.1 mg (yield 8.9%) with liver homogenate and kidney homogenate as the enzyme source respectively. The crystals melted at 135–138 °C, and this was not changed by admixture with the isolated urinary compound. Analytical data found: C, 41.63; H, 4.72; N, 10.58%. The f.a.b.-mass spectrum (Figure 2c) of the product was identical with those of synthesized CIE-TL (Figure 2a) and the isolated urinary compound (Figure 2b). This product behaved identically with the isolated urinary compound on the high-voltage paper electrophoresis and had an identical i.r. spectrum. From these data, the product was determined to be CIE-TL.

CIE-TA was found in the 200–260 ml fractions of the 0.1 M acetic acid eluates and the 0–20 ml fractions of the 0.2 M acetic acid eluates obtained as described above, and crystals were obtained in a yield of 18.2% and 9.8% with liver homogenate and kidney homogenate respectively. In addition, products with various mR_{TLA} values around 0.3–0.5 were found mainly in the 0.5 M and 2 M acetic acid eluates. These products were combined. Signals at m/z 259 and 215 were found on the f.a.b.-mass spectrum of the combined products. After the products had been treated with 6 M HCl at 100 °C for 2 h, f.a.b.-m.s. was again carried out. The signal at m/z 215 was weakened and the signal at m/z 259 had disappeared; instead, a new signal assigned as MH^+ of CIE-TA was found at m/z 231. The signals at m/z 259 and 215 may be due to CIE-TP (Figure 1) and S-[2-carboxy-1-(1*H*-imidazol-4-yl)ethyl]-2-thioacetaldehyde (CIE-TAal) respectively as discussed below.

Degradation of the smaller amount of CIE-Cys (25 μ mol) with rat kidney homogenate produced CIE-TL and CIE-TA in yields of 8.2% and 12% respectively.

DISCUSSION

From these results, one of the physiological precursors of the urinary compounds CIE-TL and CIE-TA was demonstrated to be CIE-Cys. CIE-Cys is formed by enzymic degradation of CIE-SG possibly via the intermediate CIE-Cys-Gly, catalysed by γ -glutamyltransferase (EC 2.3.2.2) and dipeptidase (EC 3.4.13.11) as described previously (Kinuta et al., 1993).

From the chemical structures of CIE-Cys and CIE-TL, it was postulated that CIE-TP is a metabolic intermediate between the two compounds. During the metabolism of L-cysteine, 3-mercaptopyruvic acid is formed by oxidative deamination with L-amino acid oxidase (EC 1.4.3.2) or by the transamination pathway (3-mercaptopyruvate pathway) (Ubuka et al., 1992a,b; Meister et al., 1954) which is catalysed by L-cysteine aminotransferase identified with aspartate aminotransferase (EC 2.6.1.1) in rat liver mitochondria (Ubuka et al., 1978) and cytosol (Akagi, 1982). 3-Mercaptopyruvic acid is further metabolized to L-3-mercaptolactic acid by the action of L-lactate dehydrogenase (EC 1.1.1.27) (Ohta and Ubuka, 1989), although 3-mercaptopyruvic acid is unstable *in vitro* (Cooper et al., 1982). Therefore it is possible that CIE-Cys is catabolysed by these enzymes to give CIE-TL via the proposed intermediate CIE-TP. 3-Mercaptopyruvic acid is unstable in acid, decomposing in part to 2-mercaptoacetic acid by decarboxylation to 2-mercaptoacetaldehyde and then oxygenation. Acid treatment of the products of enzymic degradation of CIE-Cys resulted in the disappearance and weakening respectively of signals at m/z 259 and 215 on the f.a.b.-mass spectrum as described above; instead, the signal assigned as MH^+ of CIE-TA was observed at m/z 231. If CIE-TP (M_r , 258) is decomposed, like 3-mercaptopyruvic acid, by HCl, CIE-TAal (M_r , 214) is formed and this may be followed in part by oxygenation to CIE-TA (M_r , 230). An attempt to isolate the compounds giving signals at m/z 259 and 215 was unsuccessful because of their instabilities in acid and air.

CIE-TL has two asymmetric carbon atoms, an α -carbon on the thiolactate moiety and a β -carbon on the skeletal imidazolylpropanoate part of the molecule. The isolated urinary compound may have an *R* configuration on the α -carbon because of the L-cysteine moiety of the CIE-Cys molecule. The configuration on the β -carbon has not been specified, although CIE-Cys used in the present work was made by the addition of *trans*-urocanic acid to L-cysteine. In epidermis, u.v. light isomerizes *trans*-urocanic acid to the *cis* form (Anglin et al., 1961) and epidermal *cis*-urocanic acid may participate in the formation of CIE-Cys and CIE-SG under conditions of sunlight irradiation as discussed previously (Kinuta et al., 1991); the content of *cis*-urocanic acid in the body, however, is even lower than that of the *trans* isomer.

This work was supported in part by a Grant-in-Aid for Scientific Research from the Ministry of Education, Science and Culture of Japan.

REFERENCES

- Akagi, R. (1982) *Acta Med. Okayama* **36**, 187–197
- Anglin, J. H., Jr., Bever, A. T., Everett, M. A. and Lamb, J. H. (1961) *Biochim. Biophys. Acta* **53**, 408–409
- Barden, H. P. and Pathak, M. A. (1967) *J. Invest. Dermatol.* **48**, 11–17
- Cooper, A. J. L., Hber, M. T. and Meister, A. (1982) *J. Biol. Chem.* **257**, 816–826
- Hais, I. M. and Štrych, A. (1968) *Experientia* **24**, 231–232
- Kinuta, M., Yao, K., Masuoka, N., Ohta, J., Teraoka, T. and Ubuka, T. (1991) *Biochem. J.* **275**, 617–621
- Kinuta, M., Ubuka, T., Yao, K., Futani, S., Fujiwara, M. and Kurozum, Y. (1992a) *Biochem. J.* **283**, 39–40
- Kinuta, M., Masuoka, N., Yao, K., Ohta, J., Yoshida, S., Futani, S. and Ubuka, T. (1992b) *Amino Acids* **1**, 259–262
- Kinuta, M., Ubuka, T., Yao, K., Yamada, S., Yukihiro, K. and Tomozawa, M. (1993) *Biochim. Biophys. Acta* **1157**, 192–198
- Kral, J. A., Zenisek, A., Štrych, Z. and Hais, I. M. (1968) *Cas. Lek. Cesk.* **107**, 302–307
- Macpherson, H. T. (1946) *Biochem. J.* **40**, 470–481
- Meister, A., Fraser, P. E. and Tice, S. V. (1954) *J. Biol. Chem.* **206**, 561–575
- Ohta, J. and Ubuka, T. (1989) *Acta Med. Okayama* **43**, 89–95
- Pauly, H. (1904) *Hoppe-Seyler's Z. Physiol. Chem.* **42**, 508–518
- Scott, I. R. (1981) *Biochem. J.* **194**, 829–838
- Toennis, G. and Kolb, J. J. (1951) *Anal. Chem.* **23**, 823–826
- Ubuka, T. (1962) *J. Biochem. (Tokyo)* **52**, 440–443

Ubuka, T., Umemura, S., Yuasa, S., Kinuta, M. and Watanabe, K. (1978) *Physiol. Chem. Phys.* **10**, 483–500

Ubuka, T., Ohta, J., Yao, W.-B., Abe, T., Teraoka, T. and Kurozumi, Y. (1992a) *Amino Acids* **2**, 143–155

Ubuka, T., Ohta, J., Akagi, R., Hosaki, Y., Ishimoto, Y., Kiguchi, S., Ikeda, T. and Ishino, K. (1992b) *Amino Acids* **3**, 243–252

Wadia, A. S., Sule, S. M. and Mathur, G. P. (1975) *Indian J. Exp. Biol.* **13**, 234–237

Zannoni, V. G. and La Du, B. N. (1963) *Biochem. J.* **88**, 160–162

Received 1 June 1993/2 August 1993; accepted 20 August 1993