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Almost all about citrulline in mammals

Review Article

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Summary. Citrulline (Cit, $C_6H_{13}N_3O_3$), which is a ubiquitous amino acid in mammals, is strongly related to arginine. Citrulline metabolism in mammals is divided into two fields: free citrulline and citrullinated proteins. Free citrulline metabolism involves three key enzymes: NO synthase (NOS) and ornithine carbamoyltransferase (OCT) which produce citrulline, and argininosuccinate synthetase (ASS) that converts it into argininosuccinate. The tissue distribution of these enzymes distinguishes three "orthogonal" metabolic pathways for citrulline. Firstly, in the liver, citrulline is locally synthesized by OCT and metabolized by ASS for urea production. Secondly, in most of the tissues producing NO, citrulline is recycled into arginine via ASS to increase arginine availability for NO production. Thirdly, citrulline is synthesized in the gut from glutamine (with OCT), released into the blood and converted back into arginine in the kidneys (by ASS); in this pathway, circulating citrulline is in fact a masked form of arginine to avoid liver captation. Each of these pathways has related pathologies and, even more interestingly, citrulline could potentially be used to monitor or treat some of these pathologies. Citrulline has long been administered in the treatment of inherited urea cycle disorders, and recent studies suggest that citrulline may be used to control the production of NO. Recently, citrulline was demonstrated as a potentially useful marker of short bowel function in a wide range of pathologies. One of the most promising research directions deals with the administration of citrulline as a more efficient alternative to arginine, especially against underlying splanchnic sequestration of amino acids. Protein citrullination results from post-translational modification of arginine; that occurs mainly in keratinization-related proteins and myelins, and insufficiencies in this citrullination occur in some auto-immune diseases such as rheumatoid arthritis, psoriasis or multiple sclerosis.

Keywords: Citrulline metabolism – Urea cycle – Citrullinated proteins – Nitric oxide metabolism – Argininosuccinate synthetase – Ornithine carbamoyltransferase

1 Introduction

Citrulline takes its name from the Latin for watermelon, *Citrullus vulgaris*, which contains large amounts of this amino acid.

Until recently, citrulline (Cit) attracted relatively little interest, almost certainly because it is a non-protein amino acid. However, recent studies have underlined the importance of this amino acid in both cellular metabolism and in monitoring of organ functionality. It is therefore useful to provide a summary of the properties and metabolism of this amino acid. For convenience, this paper will be limited to the metabolism and role of citrulline in mammals, and we only briefly refer to certain specificities in other kingdoms (plants and bacteria) where appropriate.

We will start by outlining the main physical and chemical properties of citrulline, which are necessary to understand its metabolism in biological systems and construct structural or metabolic models involving the citrulline molecule. We will go on to discuss the implication of citrulline in mammal metabolism and conclude with the involvement of citrulline in pharmaceutical and medical arts.

2 Physical properties

Citrulline (CAS: 372–75–8) is a colourless solid at ambient temperature and pressure. Its melting point is 222°C. It is an α-amino acid with an asymmetric carbon; hence, it presents two enantiomers. Like most amino acids, its natural form is the L form (dextrogyrate, $[\alpha_D^{20}] = 3.7°$, with absolute configuration *S*). Its developed formula is presented in Fig. 1.

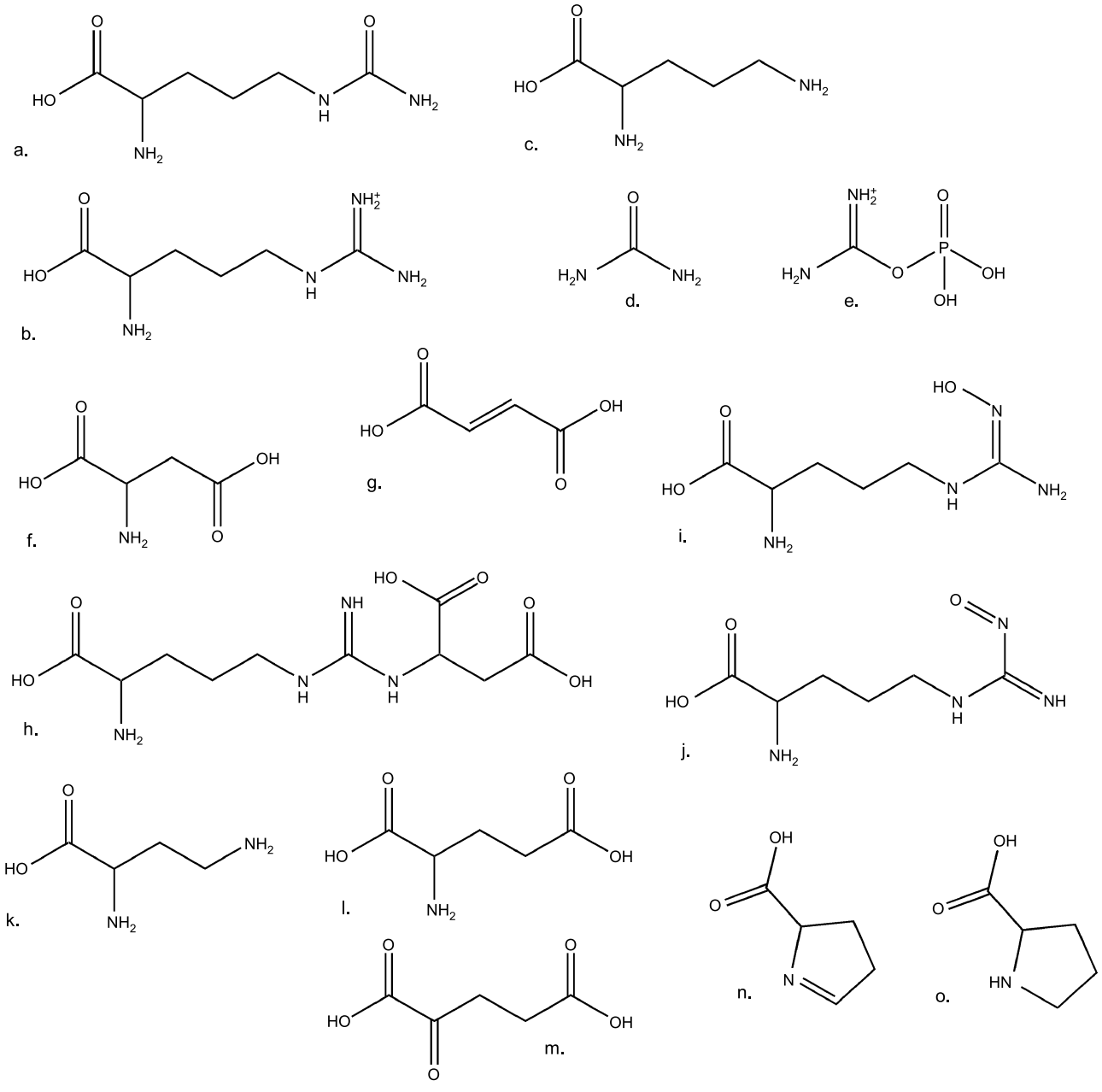

Fig. 1. Developed formula of citrulline (**a**) and related compounds. Urea cycle-related compounds: arginine (**b**), ornithine (**c**), urea (**d**), carbamoyl phosphate (**e**), aspartic acid (**f**), fumaric acid (**g**), argininosuccinate (**h**). NO synthesis intermediates: (**i**) and (**j**). Citrulline biosynthesis: glutamine (**k**), glutamic acid (**l**), α-ketoglutarate (**m**, 2-oxoglutarate), Δ^1-L-pyrroline-5-carboxylate (**n**, Δ^1-P5C) and proline (**o**)

It also exists as salts of citrullinium, its cationic form, the most frequent being chlorhydrate, which is also dextrogyrate ($[\alpha_D^{20}] = 17.9°$).

2.1 Crystal structure

The crystalline structure of citrulline and various citrullinium salts are known. Their characteristics are set out in Table 1. Each unit cell contains two citrulline molecules (or citrullinium cations) presenting hydrogen bond interactions. A comparison of distances and angles, summarized in Table 2, indicates that all these structures are comparable, except for the orientation of the ureide group. A least square fit between the crystal structure conformations is illustrated in Fig. 2. Two different conformations arise from the dihedral rotation around the C_α–C_β bond,

Table 1. Crystallographic parameters of the known crystals of citrulline and citrullinium salts

Salt	System	Space group	Unit cell parameters				Reference
			a	*b*	*c*	β	
Zwitterion	Monoclinic	$P2_1$	9.162(5)	5.143(3)	8.969(3)	95.81(2)	Toffoli et al. (1987)
Dihydrate	Orthorhombic	$P2_12_12_1$	4.671(4)	13.07(1)	16.40(1)	90	Xianglin et al. (1985)
			16.479(5)	13.134(3)	4.6779(8)	90	Toffoli et al. (1986)
Chloride	Monoclinic	C_2	17.86(2)	5.09(1)	11.77(2)	106.8(2)	Naganathan and Venkatesan (1971)
			18.089	5.150	11.918	106.9	Ashida et al. (1972)
Perchlorate	Orthorhombic	$P2_12_12_1$	5.1113(1)	11.3497(2)	19.3853(3)	90	Sridhar et al. (2002)
Malate	Monoclinic	$P2_1$	8.934(3)	5.368(1)	14.377(4)	91.96(3)	Toffoli et al. (1988)

Figures in brackets indicate the precision of the data

Table 2. Comparison of molecular distances (Å) and angles (°) in crystalline citrulline

Reference	Bond lengths										
	O_2–C_1	C_1–O_1	C_1–C_2	C_2–N_1	C_2–C_3	C_3–C_4	C_4–C_5	C_5–N_2	N_2–C_6	C_6–O_3	C_6–N_3
Toffoli et al. (1987)	1.16	1.24	1.52	1.45	1.49	1.57	1.53	1.51	1.36	1.25	1.39
Toffoli et al. (1986)	1.17	1.25	1.49	1.46	1.49	1.56	1.53	1.56	1.31	1.27	1.38
Naganathan and Venkatesan (1971)	1.21	1.23	1.48	1.43	1.49	1.41	1.53	1.33	1.32	1.17	1.33
Ashida et al. (1972)	1.22	1.26	1.50	1.47	1.51	1.43	1.55	1.37	1.31	1.18	1.32
Sridhar et al. (2002)	1.22	1.28	1.51	1.48	1.51	1.45	1.51	1.38	1.34	1.30	1.39
Toffoli et al. (1988)	1.14	1.25	1.49	1.44	1.50	1.58	1.51	1.51	1.34	1.26	1.40

	Dihedral angles					
	C_3–C_2–C_1–O_1	C_4–C_3–C_2–C_1	C_5–C_4–C_3–C_2	N_2–C_5–C_4–C_3	C_4–C_5–N_2–C_6	N_3–C_6–N_2–C_5
Toffoli et al. (1987)	111.827	166.219	175.972	67.616	105.350	165.706
Toffoli et al. (1986)	101.624	176.617	181.496	79.672	240.917	184.285
Naganathan and Venkatesan (1971)	127.775	303.911	189.231	177.712	91.067	170.352
Ashida et al. (1972)	126.798	303.025	188.407	180.178	91.996	168.614
Sridhar et al. (2002)	135.489	304.007	183.439	181.944	164.613	350.859
Toffoli et al. (1988)	102.066	165.763	177.997	69.627	99.493	170.897

The coordinates of the structure by Xianglin et al. (1985) correspond to D-citrulline and are not included in the table

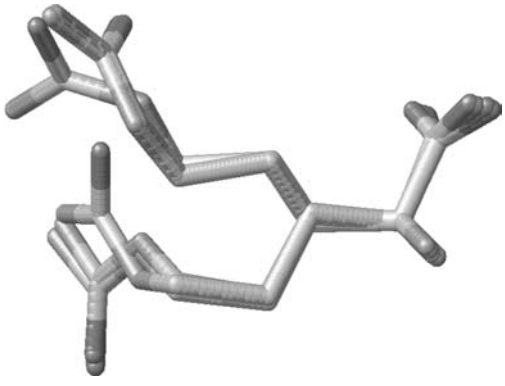

Fig. 2. Mass-weighted least squares fit of the 6 crystalline citrulline conformations. See Table 1 for references

one for a dihedral of ~170° and another for a dihedral of ~300°. Three crystal structures adopt the first conformation and three others the second conformation.

In order to study the conformations of the citrulline molecule in solution, we used the Gromacs package (Lindahl et al., 2001; Berendsen et al., 1995) to perform a 10 ns molecular dynamics simulation of a zwitterionic citrulline molecule solvated with 1397 SPC water molecules in a cubic box with ~35 Å edges. Simulation was performed using particle mesh Ewald electrostatics (Essmann et al., 1995) and a weak coupling algorithm for temperature and pressure (Berendsen et al., 1984). Clustering of the conformations produced by the dynamics run led to the two

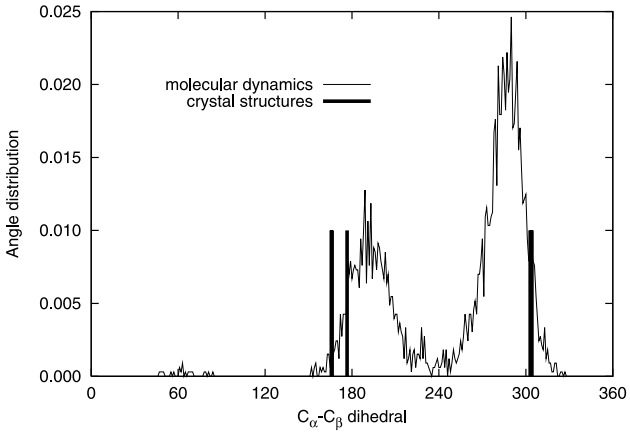

Fig. 3. Distribution of the C_α–C_β dihedral angle computed from molecular dynamics simulation and values observed in crystal structures

conformations observed in solid state, as can be seen from the C_α–C_β dihedral angle distribution (Fig. 3).

2.2 Spectroscopic properties

Nuclear magnetic resonance (NMR) spectra have been described in the literature for hydrogen, carbon and nitrogen (see Table 3 for references). The proton spectra are recorded in D_2O, therefore exchangeable protons cannot be detected, but the water peak appears at 4.8 ppm. Other shifts are summarized in Table 3.

Infrared spectra, recorded in Nujol, are available in commercial compilations (Collective, 1997) for both racemic mixture and L enantiomer form.

Table 3. Chemical shifts for proton (t = triplet, m = multiplet), nitrogen and carbon in citrulline

	Shift (ppm)			
	^{1}H		^{13}C	^{15}N
	Pouchert (1983)	Ganadu et al. (1991)		Suzuki et al. (1974)
COOH	ex.	ex.	177.3	
CH	3.8	3.65t	56.0	
NH_3^+	ex.	ex.		332.8
CH_2	1.5	1.77 m	30.3	
CH_2	1.5	1.46 m	46.9	
CH_2	3.2	3.03t	27.3	
NH	ex.	ex.		283.6
CO			163.6	
NH_2	ex.	ex.		295.9

Proton spectra were recorded in D_2O, therefore exchangeable protons (noted ex.) cannot be detected. Carbon shifts are given with TMS as reference

3 Chemical properties

Citrulline ($C_6H_{13}N_3O_3$) is an α-amino acid with a molar mass of $175\,g \cdot mol^{-1}$. Its chemical properties result not only from the amino acid function but also from the terminal ureide group of the aliphatic chain which substitutes the α-carbon.

3.1 Properties in solution

3.1.1 Solubility

Because of its polar lateral chain, citrulline is relatively soluble in water but barely soluble in methanol and ethanol. This can be verified by its exchange constant between octanol and water: $\log P = -3.19 \pm 0.11$ (indirect determination from thin-layer chromatography, Pliška et al., 1981).

3.1.2 Bronsted acidobasicity

As with all amino acids, citrulline presents the two acidities of the carboxylic acid (pK_a around 2.4) and amine (pK_a around 9.4) groups. Various pK_a values depending on experimental conditions have been cited in the literature: Table 4 provides a summary. Other acidities are too small to be observed in water, and will not be mentioned here.

Since citrulline is a diacid, it is also important to consider micro-acidities taking into account the various

Table 4. Various literature values for the acidity constants of citrulline in water, with corresponding conditions (*T*: temperature; *I*: ionic strength)

T (°C)	*I*	pK_1	pK_2	Reference
25	0.1 mol/kg		9.71	Clarke and Martell (1970)
25	0.1 mol/kg		9.41	Ellenbogen (1952)
25	0.1 mol/kg	2.12	8.64	Trikha et al. (1968)
25	0.1 mol/L	2.275 ± 0.005	9.349 ± 0.002	Ganadu et al. (1991)
25	0.1 mol/L	2.43	9.43	Steglich et al. (2000)
25	ideal		9.72	Tewari et al. 1993
25		2.32	9.30	Lide (2004)
20	0.01 mol/kg		9.69	Perkins (1953)
	0.3 mol/L	2.30	9.48	Noszál and Kassai-Tánczos (1991)
	0.5 mol/L	2.33	9.49	Noszál and Kassai-Tánczos (1991)
	1.0 mol/L	2.30	9.50	Noszál and Kassai-Tánczos (1991)
	1.5 mol/L	2.31	9.55	Noszál and Kassai-Tánczos (1991)
		2.43	9.41	Budavari et al. (2001)

Blank values were not determined in the given reference. pK_2 from Trikha et al. (1968) is surprisingly low and may be a typo in the text, to be read 9.64. Tewari et al. (1993) also give the thermodynamic parameters of the protonation: $\Delta_r H_m^o = 49.8\,kJ/mol$, $\Delta_r S_m^o = -19\,kJ/mol/K$

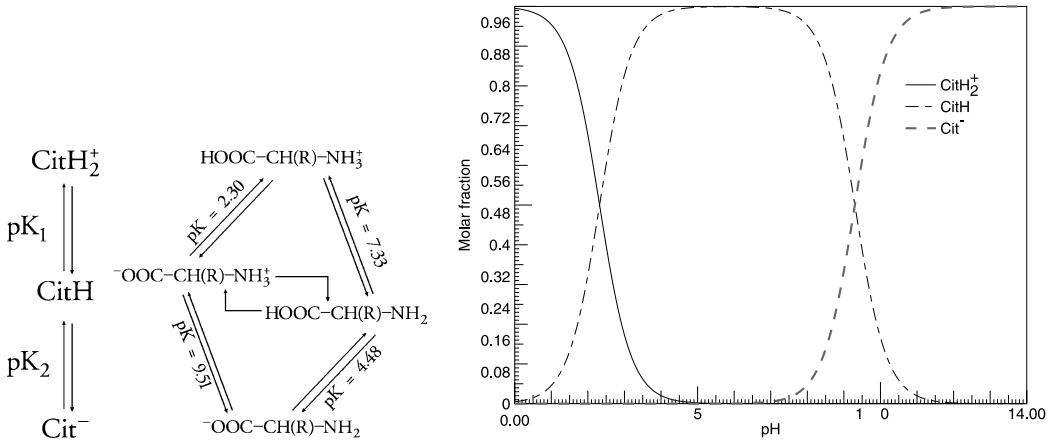

Fig. 4. Predominant species of citrulline in aqueous solution

deprotonation steps. The micro-acidity constants are known (Noszál and Kassai-Tánczos, 1991) and are presented in Fig. 4, which also summarizes the distribution of the different species in water for the complete pH range. Given these micro-acidities, the predominant neutral form is the zwitterion (more than 99.9%), which justifies the use of macroscopic constants in common usage. This diagram clearly shows that, under physiological conditions, citrulline is present almost entirely as a zwitterion.

3.1.3 Complexation properties

At alkaline pH, both the carboxylic and amine groups can act as Lewis donors, which means they can complex

Table 5. Literature data on the complexation of metallic cations by citrulline. Only binary complexes are reported (*I*: ionic strength (in mol/L))

Cation	*I* (M)	*T* (°C)	Log K_f			Method	Reference
			ML	ML_2	ML_3		
Be^{2+}	0.01?	20	13.0			Polarimetry	Perkins (1953)
Ca^{2+}	0.1	25	1.65	1.85		Potentiometry	Clarke and Martell (1970)
Cd^{2+}	0.1		3.47	6.41		pH-metry	Trikha et al. (1968)
		20		7.3		Polarimetry	Perkins (1953)
	0.2	23	4	7	8.94	Polarography	Suyan et al. (1979)
	0.2	30	8.88	6.35	8.82	Polarography	Suyan et al. (1979)
Co^{2+}	0.1		3.84	6.83		pH-metry	Trikha et al. (1968)
	0.1	25	3.94	6.48*		Potentiometry	Clarke and Martell (1970)
Cr^{3+}							Samitz and Katz (1964)
Cu^{2+}	0.1		7.23	13.27		pH-metry	Trikha et al. (1968)
	0.1	25	7.92	14.39*		Potentiometry	Clarke and Martell (1970)
Hg^{2+}		20		18.8		Polarimetry	Perkins (1953)
	0.1	25	6.02	10.95		Potentiometry	Clarke and Martell (1970)
Mg^{2+}	0.1	25	1.66	1.86		Potentiometry	Clarke and Martell (1970)
Mn^{2+}	0.1	25	1.59	*		Potentiometry	Clarke and Martell (1970)
Ni^{2+}	0.1		4.82	8.67		pH-metry	Trikha et al. (1968)
	0.1	25	5.10	9.10*		Potentiometry	Clarke and Martell (1970)
Pb^{2+}	0.1		4.47			pH-metry	Trikha et al. (1968)
Pd^{2+}	0.1	25		16.23 ± 0.02		Potentiometry	Ganadu et al. (1991)
UO_2^{2+}	0.1		6.85			pH-metry	Trikha et al. (1968)
Zn^{2+}	0.1		4.22	7.64		pH-metry	Trikha et al. (1968)
	0.1	25	4.13	*		Potentiometry	Clarke and Martell (1970)
	0.01?			8.7		Polarimetry	Perkins (1953)

Method = the method used to detect the complex formation and, where appropriate, to estimate the formation constant. Blank values were not determined in the given reference. A star indicates that a precipitate was obtained during the experiments. The results from Trikha et al. (1968) should be taken with caution, due to the unusually small value of the second acidity constant obtained by these authors (see Table 4)

metallic cations. The lateral chain is much less complexing, since non-binding electrons of the nitrogen atoms are conjugated with the double bond. Hence, complexes formed by citrulline are similar to those formed by other ''classical'' amino acids such as glycine or alanine, and are able to yield salts in solid state. However, since no crystal structure of any citrulline complex is mentioned in the Cambridge Crystallographic Database (despite the mention of blue crystals for the copper complex in Buckingham, 1994), this coordination scheme is only supported from indirect data in solution form.

Various other complexes have been reported in aqueous solution (see Table 5 for binary complexes. One ternary complex has been reported: $[Cu^{II}(His)(Cit)]$, with $K_f = 17.67 \pm 0.02$ (Yamauchi et al., 1980)). None of these complexes have yet been reported to be of biological interest, except the Cr^{3+} complex that may be formed in skin after chromium intoxication (Samitz and Katz, 1964).

3.2 Reactivity

3.2.1 Amino acid reactivity

Citrulline presents the common reactivity of the α-amino acid family. In particular, it can form peptide bonds; hence it can therefore be present in proteins. However, since there is no known codon in the genetic table for this amino acid, its presence in a protein must always result from a post-translational modification of the protein. The biological role of citrullinated proteins will be discussed in paragraph 8.

Note that the biochemical conversion from arginyl to citrullyl residue can be applied during experimental studies by replacing a potentially active arginine in a protein by a citrulline, and then studying the effect of this artificial modification on the protein's activity. This technique has been successfully applied in various proteins: see e.g. Imparl et al. (1995) for an interaction between two proteins, or Eronina et al. (1996) for the allosteric regulation of an enzyme.

3.2.2 Ureide group reactivity

As can be seen from its formula (Fig. 1), citrulline can be distinguished from the other amino acids by the presence of the ureide group. It is thus not surprising that the biological role of citrulline is based on the chemical reactivity of this group, and especially its close relation to the functional group of arginine.

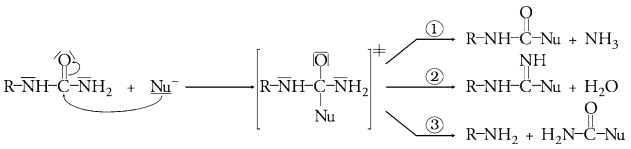

Fig. 5. Reactivity of the citrulline ureide group. These various possible reactions form the basis of the biological use and synthesis of citrulline

This reactivity is due to the strongly electrophilic carbon atom in the ureide group, with its electrons strongly attracted by the surrounding nitrogen and oxygen atoms. Hence, a relatively weak nucleophilic compound R can react on this carbon to form the unstable intermediate $N_2C(R)O^-$. This intermediate will stabilize by expelling one of its four ligands: either NH_3, R (no reaction), $R–NH_2$ (leading to the formation of ornithine) or water. All these reactions can only occur in the presence of proton acceptors and donors, which may mean water or, in most cases, convenient amino acids from the active site of enzymes. These reactions are summarized in Fig. 5.

Biologically speaking, the most important reaction is the last one, with aspartate as nucleophilic compound (''R''); the reaction produces argininosuccinate and constitutes a step in the urea cycle (see Sections 4.3.6 and 6.3).

All these reactions can also operate in the opposite direction, thus giving citrulline as end product. This is how citrulline is prepared industrially from arginine, with water as nucleophile and ammonia as leaving group. Biologically, the leaving group can be either a phosphate (in the urea cycle, after condensation of ornithine with carbamoyl phosphate, see Sections 4.1.5 and 6.3) or nitric oxide, and this is, in fact, the biological synthesis pathway for nitric oxide (see Section 4.2).

3.3 Citrulline assay

Several methods have been proposed for quantifying citrulline in biological samples. This section will simply give a brief presentation of these techniques, and we will refer interested readers to the original literature for more information. Basically, the methods can be split into two families: generic amino acid quantification methods and citrulline-specific methods.

3.3.1 Generic methods of amino acid quantification

These methods rely on the detection of the amino acid function, regardless of the side chain. Detection can then be performed by ninhydrin staining (the most popular method), by orthophtaldialdehyde or by phenylthiocarbamyl. Since they are non-specific, these methods must be

Table 6. Concentrations of citrulline in various tissues (CSF: cephalo-spinal fluid). Concentrations are expressed in $\mu mol \cdot L^{-1}$, except for the brain where they are in $\mu mol \cdot g^{-1}$. Results are given as means ± SD or range. IEC: Ion Exchange Chromatography, RPC: Reversed Phase Chromatography, HPLC: High Pressure Liquid Chromatography; ESI: detection with electrospray mass spectrometry, Nin: detection with ninhydrin, OPA: *o*-phtaldialdehyde detection, PTC: phenylthiocarbamyl detection; DAM: diacetylmonoxime method; *: compilation of values from various sources. See Lepage et al. (1997) for details on effect of age on plasma concentration

Biological fluid or tissue	Organism	Concentration	Method	Reference
Plasma	Rat (adult)	70 ± 8	RPC	Mistry et al. (2002)
	Rat (aged)	171 ± 23	RPC	Mistry et al. (2002)
	Human (newborn)	16 ± 5	IEC	Dickinson et al. (1965)
		14–32	IEC–Nin	Lepage et al. (1997)
	Human (child)	12–30	IEC	Scriver et al. (1965)
		17–39	IEC–Nin	Lepage et al. (1997)
	Human (adult)	20–50	IEC	Pappas et al. (2002)
		27–38	Isotopes	Castillo et al. (1993)
		24 ± 5	RPC–OPA	Ziegler et al. (1992)
		25 ± 2	Isotopes	Lau et al. (2000)
		28 ± 11	RPC–PTC	Feste (1992)
		29 ± 4	IEC–Nin	Muscaritoli et al. (1999)
		29 ± 11	IEC	Peters et al. (1969)
		31 ± 6	IEC–Nin	Wick et al. (1970)
		31 ± 7	RPC–OPA	Fekkes et al. (1995)
		32 ± 7	RPC–OPA	Zhang and Kaye (2004)
		33 ± 8	DAM	Kamoun et al. (1983)
		34 ± 1	RPC	Alteheld et al. (2004)
		35 ± 8	IEC	Perry and Hansen (1969)
		36 ± 10	HPLC–ESI	Martens-Lobenhoffer and Bode-Böger (2003)
		38 ± 8	IEC–Nin	Le Boucher et al. (1997)
Urine	Human (adult)	4 ± 4	HPLC–ESI	Martens-Lobenhoffer and Bode-Böger (2003)
		≤4	IEC	Neveux et al. (2004)
Brain	Human	0.02–0.1	*	Perry (1982)
CSF	Human	1.5–21.6	*	Wiesinger (2001)
		1.5 ± 0.5	IEC	Neveux et al. (2004)
Muscle	Human	170 ± 13	RPC	Alteheld et al. (2004)

coupled with separation methods in order to be applied in biological fluids. More than the detection step (which may be based on pre- or post-derivatization), it is thus the separation step that differentiates the methods. In practice, the most commonly used methods are based on high pressure liquid chromatography (HPLC), either using an ion exchange column or a reversed phase column (see Table 6 for references, and Neveux et al. (2004) and Alteheld et al. (2004) for discussions on the many variants of these methods).

Except for the ninhydrin reaction, all the previous detection methods rely on the fluorescence of the compound formed between the detecting compound and citrulline. This often implies the use of a pre-treatment to reduce parasitic fluorescence, but this step can be avoided by performing detection using electrospray mass spectroscopy (Martens-Lobenhoffer and Bode-Böger, 2003), which also partially ensures separation of the compounds.

3.3.2 Specific methods for citrulline assay

The presence of the ureide group makes it possible to use a more specific technique based on the quantification of urea, where citrulline acts like a substituted urea. This method, which uses diacetylmonoxim, requires urea to be removed from the sample prior to analysis, and this can be achieved through the action of a urease (Siest et al., 1968; Kamoun et al., 1983). Here again, coupling with separation techniques, such as ion-exchange chromatography, and optimization makes it possible to increase both assay times and technique sensitivity (see Knipp and Vašák, 2000). This method can also be extended to detect citrullyl residues in proteins (Sugawara et al., 1998).

Immunological techniques have also led to the development of citrulline quantification using anti-citrulline antibodies. Specific anti-L-citrulline polyclonal antibodies are produced by coupling L-citrulline with guinea pig serum albumin, then injecting this adduct into the guinea pig (Aoki and Takeuchi, 1997). This method is especially well-suited to mapping citrulline in tissues or cells and is often used to detect the NO synthase activity, since citrulline is the second product of the NO synthase reaction (Pasqualotto et al., 1991; Rosbe et al., 1996; Keilhoff et al., 2000). However, unlike the previous methods, it does not differentiate free citrulline from the citrullyl residues in proteins, and may therefore overestimate citrulline concentrations in tissues and cells (Keilhoff and Wolf, 2003).

3.3.3 Comparison of methods

The ''generic'' methods offer the advantage of being standardized and can be fully automated. Excepting this point, the comparison of adult human plasma concentrations given in Table 6 does not suggest major differences between the results of these different methods, but this can only be confirmed by studies assaying the same samples with different methods. This has been done to compare reversed phase column with *o*-phtaldialdehyde derivatization and ion-exchange column with fluorescence detection (Fekkes et al., 1995) or ninhydrin detection (Ziegler et al., 1992), and the results of these studies have established the equivalence of these methods.

3.3.4 Methods for metabolic studies

All the quantification methods presented above give only a static view of the quantity of citrulline in a sample. To gain insight into citrulline fluxes and rate of synthesis of citrulline metabolites, other methods, based on isotope-labelled substrates, must be used.

The most commonly used method is the use of citrulline labelled with stable carbon, hydrogen or nitrogen isotopes, as performed in the metabolic studies of Castillo et al. (1993). The citrulline, its metabolites and its precursors are then detected by mass spectroscopy following separation by gas chromatography.

It appears that NMR, and more specifically ^{15}N NMR, can also track citrulline in biological samples (for example, see Legerton et al., 1981 or Lundberg and Lundquist, 2004). But, excluding one study by ^{13}C NMR on rat liver mitochondria (Nieto et al., 1992), it has not yet been applied to mammal metabolism. However, proton NMR (Burns et al., 1992; Silberstein et al., 2002) and ^{15}N NMR (Engelke et al., 2004) have already been used to detect the presence of citrulline in human tissue samples.

3.4 Natural occurrence

Given its close relationship with arginine, citrulline can be found in small amounts in almost any living organism. However, its functions differ significantly between the various organic kingdoms.

In mammals, citrulline has several functions (see Section 6 for details), and can be found in all organisms and tissues. Table 6 gives the citrulline concentrations for various tissues and cells in rats and humans. Citrullinaemia seems to vary with age, but not with alimentation (Chih-Kuang et al., 2002).

In most microorganisms, bacteria as well as parasitic nematodes, citrulline is used as an energy or carbon source. As for the various metabolic pathways of citrulline in these organisms, let us simply indicate that two enzymes are involved, ornithine carbamoyltransferase and argininosuccinate synthase (see below), that are also used in human citrulline metabolism, thus enabling these bacterial enzymes to be used as models for human enzymes.

In plants, citrulline is present at high levels in some Cucurbitacea, especially the watermelon (*Citrullus vulgaris*), and certain algae, such as *Grateloupia vulgaris*. The main role of citrulline is the transport of nitrogen (Ludwig, 1993). In a few plants, that exhibit unusually high citrulline levels (up to 300 mM), its role is believed to be a protection against oxidative stress, especially during periods of drought (Akashi et al., 2001). We will not further develop these aspects of citrulline metabolism.

4 The enzymatic basis of citrulline metabolism in mammals

Two enzymes are mainly responsible for citrulline synthesis, corresponding to the two main metabolic pathways in which it is involved: ornithine carbamoyltransferase (OCT, EC 2.1.3.3) and NO-synthase (NOS, EC 1.14.13.39, including all its iso-enzymes).

Only one enzyme, argininosuccinate synthase (ASS, EC 6.3.4.5, also known as citrulline-aspartate ligase), is capable of citrulline catabolism in mammals, and it is used in both metabolic pathways.

4.1 Citrulline synthesis: OCT (EC 2.1.3.3)

Ornithine Carbamoyl Transferase (OCT), or ornithine transcarbamylase, is found in all biological kingdoms since it is

a key enzyme in arginine synthesis and degradation: 206 sequences are registered in the SwissProt database, from prokaryotes (176 complete sequences) and eukaryotes (14 complete sequences). OCT is one of the key enzymes in the urea cycle: it catalyses the conversion of ornithine into citrulline, according to the reaction:

$$\text{Ornithine} + \text{Carbamoyl phosphate} \longrightarrow \text{Citrulline} + \text{Phosphate}$$

$$R{-}NH_2 + H_2N{-}CO{-}P_i \longrightarrow R{-}NH{-}CO{-}NH_2 + P_i$$

where P_i is a phosphate and R is the common radical of ornithine, citrulline and arginine.

This so-called ''anabolic'' OCT is involved in citrulline anabolism (arginine biosynthesis pathway), where citrulline is the product. Note that some bacterial species present an OCT variant called ''catabolic OCT'' (Baur et al., 1987), where citrulline is the substrate.

4.1.1 Reaction characteristics

Thermodynamics strongly favour the formation of citrulline: at pH between 6.7 and 7.4, with $I = 0.1\,\text{M}$ and $T = 38°\text{C}$, $\Delta_r G_m^{o\prime} = -29.8\,\text{kJ/mol}$, which gives $K' = 10^5$ (Reichard, 1957).

4.1.2 Enzyme localization

In most prokaryotes and some lower eukaryotes, the enzyme is found in the cytoplasm, but in higher eukaryotes, such as mammals, the enzyme is found in the mitochondrial matrix and functions as part of the urea cycle (Takiguchi et al., 1989). In humans, OCT is mainly present in the liver and intestinal mucosa (Ryall et al., 1985; Hamano et al., 1988; Takiguchi and Mori, 1995). This indicates that there are limits to the use of prokaryote enzymes as models for the human enzyme.

4.1.3 Structural aspects

Ornithine carbamoyltransferase is evolutionarily related to aspartate carbamoyltransferase (EC: 2.1.3.2, ATC), the two enzymes having retained similar structural conformations throughout their evolutionary development (Houghton et al., 1984). The most striking homologies correspond to regions involved in the binding of the common substrate, carbamoyl phosphate. In particular, the motif F-x-[EK]-x-S-[GT]-R-T (Prosite PS00097) is conserved in all proteins of the family, the residue in position 3 of the pattern allowing to distinguish between ATC (Glu) and OCT (Lys). The residues serine, arginine and the last threonine of the pattern have been shown by crystallography (Ke et al., 1984) to act as the binding site of the carbamoyl phosphate.

The crystal structures of the enzyme have been resolved for various organisms: *Pyrococcus furiosus*, *Escherichia coli*, *Pseudomonas aerugionosa*, humans and sheep. Anabolic OCT usually occurs as trimeric molecules with subunits of 35–40 kDa.

There are four known crystal structures of human OCT, complexed with substrate or substrate analogs: N-Phosphonacetyl-L-Ornithine (PDB code 1OTH) (Shi et al., 1998), carbamoyl phosphate and 2-amino-pentanoic acid (PDB code 1C9Y) (Shi et al., 2000) and carbamoyl phosphate (PDB codes 1FVO and 1EP9) (Shi et al., 2001). In all four crystal structures the protein fold consists of two domains of a 3-layer ($\alpha\beta\alpha$) sandwich type as shown in Fig. 6 (transcarbamylase homologous superfamily 3.40.50.1370 in the CATH classification, Aspartate/ornithine carbamoyltransferase superfamily 53671 in the SCOP classification). The first domain (PFAM signature PF02729, OTCace_N) formed mainly from the N-terminal side of the enzyme (residues 40 to 183 in the human sequence) contains the carbamoyl phosphate binding site. The second domain (PFAM signature PF00185, OTCace) on the C-terminal side (residues 186 to 342 in the human sequence) contains the ornithine binding site and is connected to the N-terminal domain by two α helices. Both binding sites are located on the hinge between the two domains. A loop (designated SMG loop and composed by residues 263–286 in the human sequence) is located underneath the hinge between the two domains and contains an invariant Ser–Met–Gly

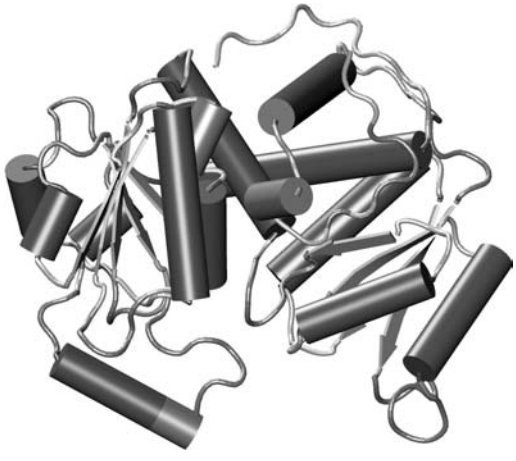

Fig. 6. The two domains of OCT, each formed by a 3-layer ($\alpha\beta\alpha$) sandwich. On the right, the N-terminal, carbamoyl phosphate binding domain, on the left, the C-terminal, ornithine binding domain. Image generated from Rasmol from the 1C9Y PDB file data

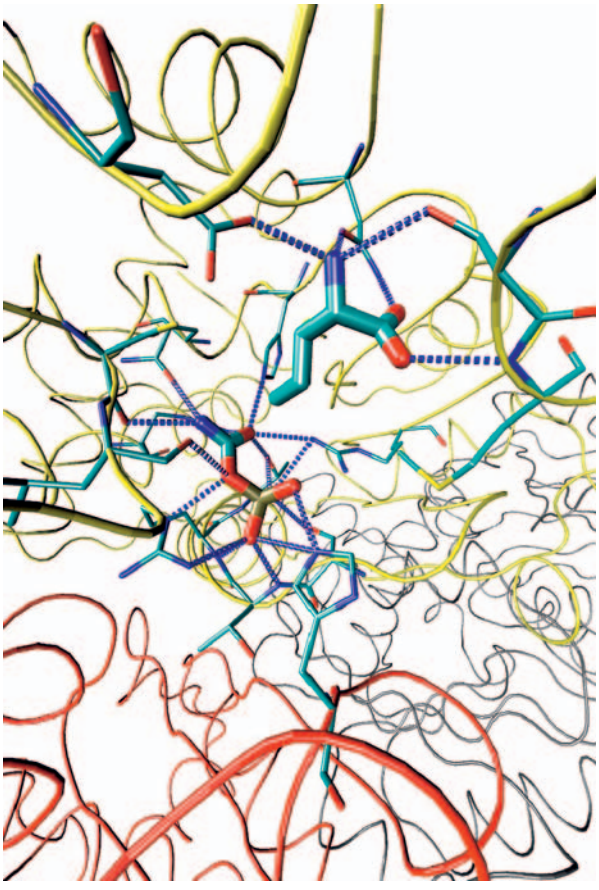

Fig. 8

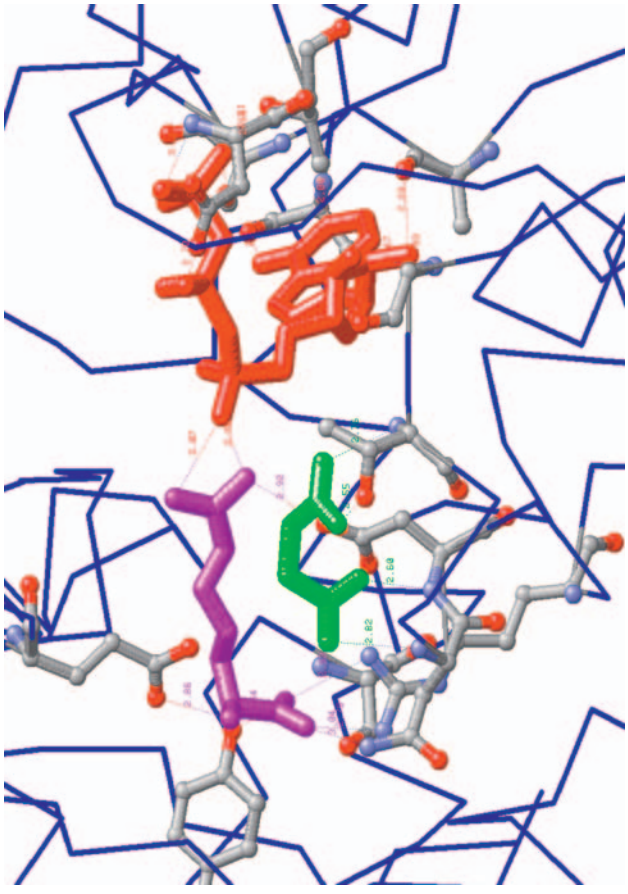

Fig. 13

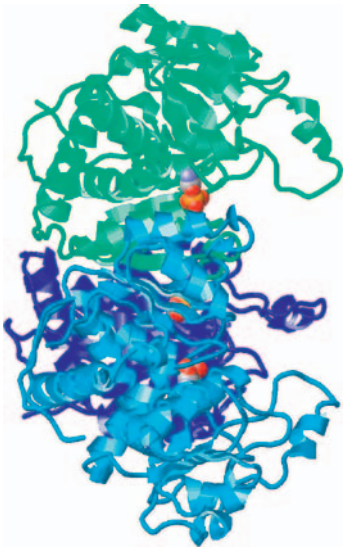

Fig. 7

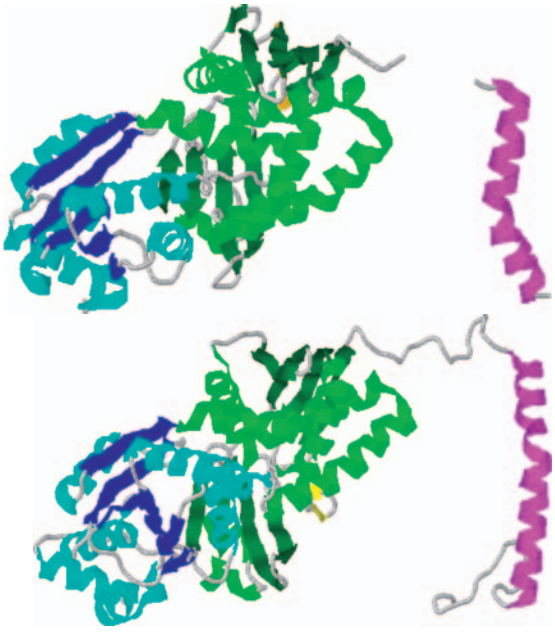

Fig. 10

pattern in all OCTases. Quaternary assembly is in the form of a homotrimer (Fig. 7), the active site being formed on the interfaces between two monomers (the trimer thus having three active sites).

4.1.4 Substrate binding

The binding of the two different substrates, i.e. carbamoyl phosphate and L-ornithine, can be deduced from crystal structures (Fig. 8). The binding site for carbamoyl phosphate is located on the N-terminal side of the enzyme. The primary nitrogen and the carbonyl oxygen of carbamoyl phosphate are coordinated to residues Gln^{171}, Cys^{303}, Arg^{330}, Thr^{93}, Arg^{141} and His^{168}. The invariant pattern residues Ser^{90}, Thr^{91}, Arg^{92} and Thr^{93} together with Arg^{141} from one subunit form hydrogen bonds to the phosphate group. Phosphate coordination is completed by the residue His^{117} from the adjacent subunit. The complex is further stabilized by a $\pi-\pi$ stacking interaction with His^{168} and some further van der Waals interactions.

L-Ornithine binding is deduced from the binding of the substrate analog 2-amino-pentanoic acid (norvaline) or the bisubstrate complex N-Phosphonacetyl-L-Ornithine. Binding site is on the C-terminal domain involving residues Asn^{199}, Asp^{263}, Ser^{267} and Met^{268}. Furthermore, a highly conserved residue of the N-terminal domain, Lys^{88}, binds the second substrate via a water molecule. Chemical modification of this Lys^{88} yields a complete loss of enzymatic activity (Valentini et al., 1996).

An extensive hydrogen bonding network involving water molecules in the binary carbamoyl phosphate/OCT complex stabilizes the side chains of residues Asn^{199} and Asp^{263} in the correct conformations to bind the L-ornithine and form the ternary complex. One of these water molecules is suspected to occupy a putative metal binding site for Zn(II) or Cd(II) (Aoki et al., 1988; Kuo et al., 1990).

4.1.5 Mechanism of the reaction

The crystal structures of complexes between OCT and bisubstrate analogs (Shi et al., 1998) or ternary complexes (Shi et al., 2000) reveal that complexation on the two binding sites induces a closure of the two domain hinges with a movement of the above-mentioned SMG loop, which swings towards the active site to cover the hinge and interact with the second substrate. Structural comparisons between OCT and ATC indicate that their common substrate, carbamoyl phosphate, binds similarly to both enzymes, consistently with the strong sequence similarity of the N-terminal portions. Molecular recognition of the second substrate seems to rely on structural differences in the C-terminal portion, especially the differences in their loops. While in ACTases the conformational change is induced upon complexation of both substrates, in OCT most of the conformational change occurs upon complexation of only carbamoyl phosphate. The main difference between the binary OCT/carbamoyl phosphate and the bisubstrate OCT/N-Phosphonacetyl-L-Ornithine structures is the position of the SMG loop. These observations have led to a complexation mechanism being proposed. First, carbamoyl phosphate binds in a deep pocket, that is accessible because the flexible SMG loop is distant to the active site. Then, L-ornithine binds and the SMG loop moves toward the active site to interact with the L-ornithine. In this ternary complex, carbamoyl phosphate is completely buried by the second substrate and the SMG loop. When the reaction is completed, the loop moves away to release the products. This mechanism is consistent with kinetic studies predicting binding of substrates in both ATC and OCT, with carbamoyl phosphate binding first, L-aspartate or L-ornithine binding second, and carbamoyl aspartate or citrulline dissociating first, with phosphate dissociating last (Porter et al., 1969; Goldsmith and Kuo, 1993).

4.2 Citrulline synthesis: NOS (EC 1.14.13.39)

The NO-synthase enzymes are responsible for the synthesis of NO and this reaction occurs in all tissues. There are three different NOS families that differ in their level of expression and their localization: nNOS is mainly present in neural cells, iNOS in macrophages and eNOS in

Fig. 7. OCT homotrimer. Substrate carbamoyl phosphate in CPK colours. Image generated from Rasmol from the 1C9Y PDB file data

Fig. 8. Substrate fixation in OCT. Carbamoyl phosphate and norvaline bound to the OCT chain (in yellow). The histidine residue on the left of carbamoyl phosphate is contributed by the adjacent subunit (in red). Image generated from Rasmol from the 1C9Y PDB file data

Fig. 10. View of the two ASS crystalline structures. From PDB files, see Lemke and Howell (2001) and Goto et al. (2002). On the left, structure of *E. coli* ASS; on the right, structure of *T. thermophilus* ASS. Only monomers are represented

Fig. 13. Substrate fixation site in ASS. ANP (ATP analog) in red, arginine in violet, succinic acid in green. Image generated from Rasmol from the 1KOR PDB file data

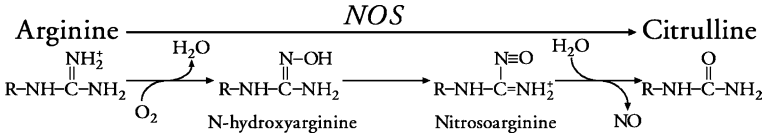

Fig. 9. Mechanism of the NO biosynthesis by NO synthases

endothelial cells. All these enzymes share a common mechanism for synthesizing NO from arginine with release of citrulline, following the reaction shown in Fig. 9, and require $NADP^+$, FMN and biopterin as cofactors (Meulemans, 2000).

There is a host of literature data covering NOS, and interested readers are invited to consult Lirk et al. (2002) or Kone (2004) for recent reviews. Since citrulline is not the primary product of this reaction (even if recycling of this citrulline is important, see Section 6.2), a detailed description of this enzyme is outside the scope of this review.

4.3 Citrulline degradation: ASS (EC 6.3.4.5)

It is a cytosolic enzyme that catalyzes the following reaction:

$$\text{Citrulline} + \text{Aspartate} + ATP \longrightarrow \text{Argininosuccinate} + \text{Pyrophosphate} + \text{AMP}$$

4.3.1 Characteristics of the reaction

In biological standard conditions, the thermodynamics of this reaction are slightly favorable to the degradation of citrulline: $\Delta_r G_m^{\circ\prime} = -2.0\,\text{kJ/mol}$ for $T = 25°\text{C}$ and $\text{pH} = 6.91$, with an ionic strength of 0.01 M (Schuegraf et al., 1960). This corresponds to an equilibrium constant of 2.14. Hence, as is often the case in metabolic pathways, the direction of the reaction is mainly controlled by the use of reaction products: in mammals, citrulline is the substrate either because arginase activity is high (i.e. in the liver) or arginine release is rapid (i.e. in the kidney), or NO synthase activity is high (e.g. in activated macrophages).

4.3.2 Enzyme localization

This enzyme exists in almost all organisms, because it is used by microorganisms to synthesize arginine. To date, only bacterial forms of the enzyme have been successfully crystallized and their structure resolved.

The localization of this enzyme in mammals has been detailed by Husson et al. (2003). Briefly, ASS is almost ubiquitous, with the higher levels found in the liver and the kidney while lowest levels are found in the intestine (in adults), but for a given tissue its expression can be limited to certain cell subpopulations or certain regions. Husson et al. (2003) also reviewed the regulation of the enzyme expression summarized here. This regulation essentially acts on the expression of the ASS gene, since there is neither post-translational modification nor catalytic kinetic control; only a direct inactivation of ASS was evidenced by covalent binding of NO to a cysteine residue of the ASS enzyme (Hao et al., 2004). Mechanisms of gene regulation differ greatly between tissues. Gene expression in the liver is modulated by hormones and nutritional status: glucocorticoids, glucagon and glutamine increase ASS levels, whereas insulin, growth hormone and oleic acid decrease them, and the underlying mechanisms of this modulation have not been clearly established. Also, the regulating factors in the kidney are not yet known. In endothelial cells, the regulation of ASS expression seems to be closely related to the NOS expression (Husson et al., 2003).

4.3.3 Characteristics of the enzyme gene

The ASS gene seems to be well conserved between all organisms, suggesting a common ancestor. It occupies 67 kb on chromosome 9 in humans (Carritt and Povey, 1979) and contains 16 exons (Suhr et al., 1991). The precise location of these exons was recently determined (Häberle et al., 2002). It seems that the DNA sequence can lead to a wide variety of mRNAs, due to the presence of three potential translational starting points (Pendleton et al. (2002), observation in bovine endothelial cells) and a second polyadenylation possibility (Su and Lin, 1990) leading to a 1 kb length difference in the transcripts (Su et al., 1981). In addition, the second exon undergoes an alternative splicing (Freytag et al., 1984; Suhr et al., 1991). The reasons for these variations are unknown, but given that the final coding sequence remains identical, regulation mechanisms are suspected. This regulation is also mediated through an unusual promoter which does not present the usual patterns of binding sites for the proteins involved in the response to hormone stimulation (Husson et al., 2003). Hence, the molecular basis of the hormonal regulation of ASS gene expression remains unknown. Husson et al. (2003) also present putative transcription factor sites, but experimental confirmation is yet to be achieved.

Further information on the ASS gene structure can be found in the works of Haberle et al. (2002) and Husson et al. (2003).

4.3.4 Enzyme structure

The SwissProt database lists 120 sequences from various organisms, and these sequences can be compared by alignment. We aligned all these sequences using BLAST whilst maintaining the taxonomic proximities through heuristic alignment methods. The results underlined the two families of ASS, named type 1 and type 2, but also the strong similarities between all ASS. In fact, type 1 and type 2 ASS differ mainly by differences in hydrophobicity profile and sequence length (450 aa for type 2 compared to only 400 for type 1). The difference in sequence length is due to an insertion of 3 amino acids at position 104 and a longer C-terminal chain. The type 1 family can itself be divided into two subfamilies: prokaryotic and eukaryotic enzymes. Vegetal enzymes, represented by *Arabidopsis thaliana*, are very similar to type 1 enzymes but present an additional set of about 100 amino acids on the N-terminal side. Since this

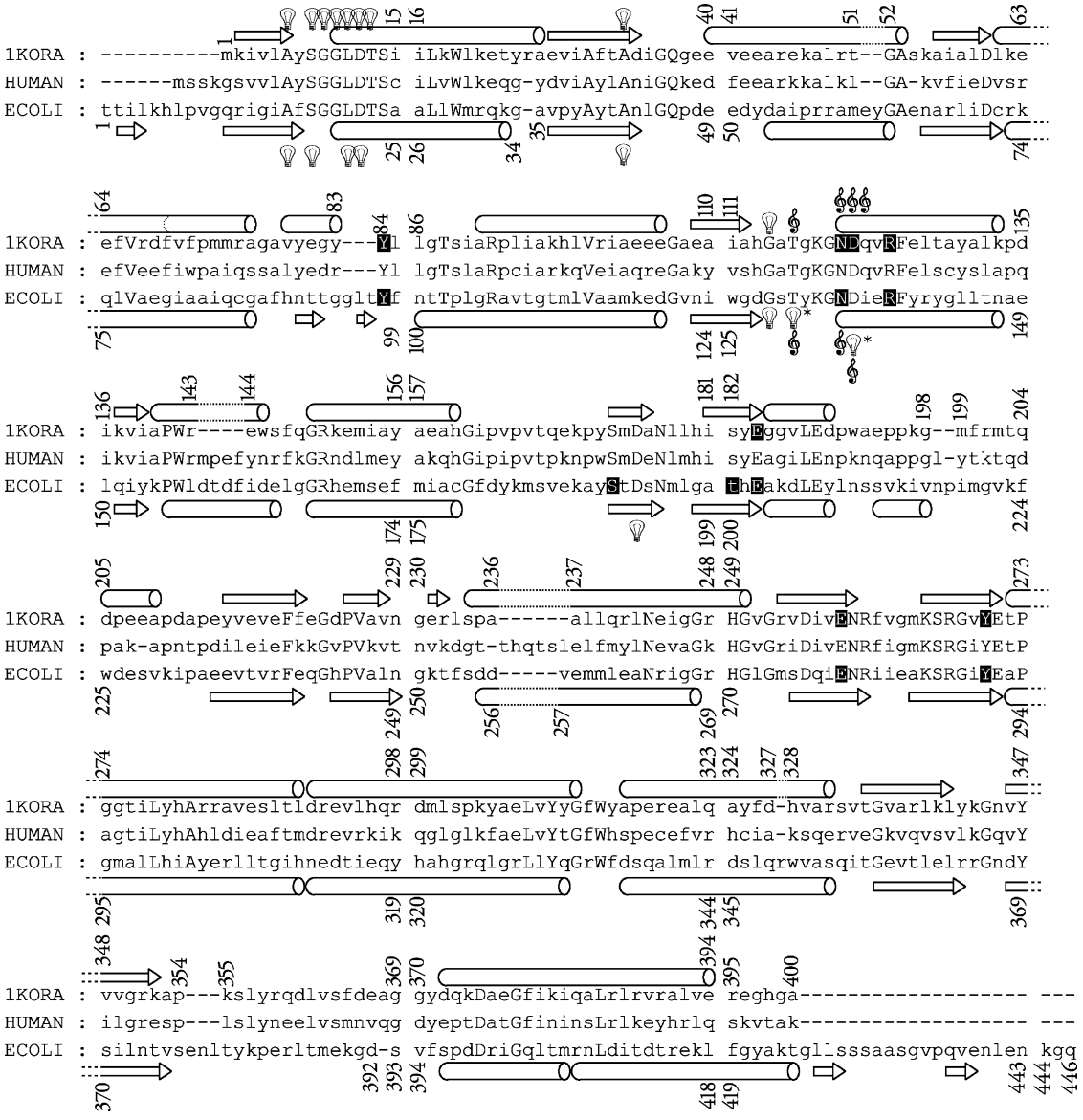

Fig. 11. Comparison of the structural domains for *E. coli* and *T. thermophilus* on the aligned sequences. The human sequence is also presented, from which secondary structure can be inferred. Arrows represent β-sheets, cylinders α-helices; both are taken from the PDB file data. Inverse video aminoacids are involved in citrulline binding: lights denote ATP-ligating aminoacids, clefs denote aspartate binding sites

enzyme was shown to be chloroplastic in plants, this additional peptide is probably a signalling peptide.

In addition to the punctual conserved positions, all the sequences present two well-conserved patterns. One is found at the beginning of the sequence and matches A-y,f-**S**-**G**-**G**-L,v-**D**-**T**-S; Prosite code PS00564 (bold indicates a strict matching; upper case indicates a strong conservation). The second pattern, about 100 aa later, matches H,d-**G**-a,c,s-**T**-G,y,f-K,m-**G**-**N**-**D**-Q,i-V,e-**R**-**F**-E,y; Prosite code: PS00565. Both patterns are characteristic of ASS; however, the first pattern is very similar to a so-called "P-loop" pattern that is found in type N ATP-pyrophosphatases. Since this pattern has been identified as binding ATP by its terminal pyrophosphate and increasing its leaving group properties when a substrate carries out a nucleophilic attack on the ATP (Tesmer et al., 1996) (first step of the substrate activation by ATP), it has been suggested that this pattern plays a similar function in ASS, and the crystal structures identified reinforce this hypothesis.

As mentioned above, two ASS enzymes have been crystallized: one from *Escherichia coli* (Lemke and Howell, 2001) (type 2, PDB entry 1K92) and the other from *Thermus thermophilus* (Goto et al., 2002) (after expression in *E. coli*; type 1 enzyme, PDB entry 1KH1). Despite approximative determination of structure (a few amino acids were not characterized in *E. coli*, and 17 residues were missing in *T. thermophilus*), the results provide valuable insight into the protein structure and the mechanism of the reaction (Fig. 10).

For both crystallized proteins, the structural unit is an asymmetric homotetramer with a second order rotation axis (C_2 group). In fact, it is a dimer of a dimer. Each monomer has a globular part and a tail (see Fig. 10), which is strongly involved in the dimerization: the monomer resembles a hook and the two monomers are placed head-to-tail, with the globular head of one of the dimers placed between the head and the hook of the other monomer. The alignment shown in Fig. 11, which presents the various structural domains, confirms the good conservation of this structure.

The structure can be better understood by the topological diagrams presented in Fig. 12, which, besides the terminal tail already cited, clearly shows the coexistence of two domains. The first domain, mainly built from the beginning of the sequence, is made of β-sheets sandwiched between α-helices. It contains the P-loop pattern and is strongly involved in the ATP binding, which is consistent with its strong similarity with the ATP-fixation domain in the N type ATP-pyrophosphatases. The second domain is involved in aspartate and citrulline binding. ASS presents a very unusual feature: both these domains

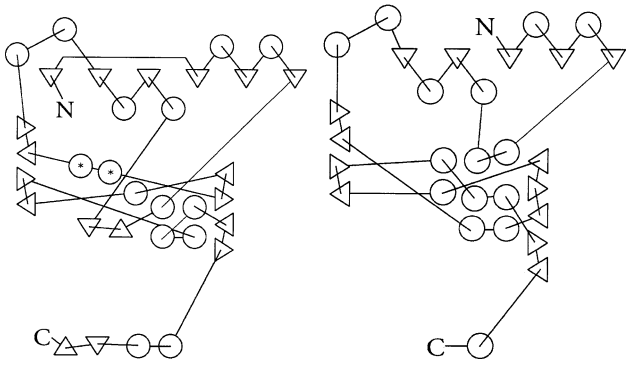

Fig. 12. Topological diagrams of *E. coli* (on left; adapted from Lemke and Howell (2001)) and *T. thermophilus* (on right). Triangles represent β-sheets, circles α-helices. The two helixes marked with stars are not in the original diagram of Lemke and Howell (2001), but are present in the PDB file

are mixed in the sequence, since two of the β-sheets (forming a hair-pin structure with a type I′ turn) and one of the α-helices of the second domain are inserted in the sequence in the middle of the ATP binding domain! This insertion sequence is longer in type 2 ASS and relatively hydrophobic, whereas in type 1 ASS it is made of polar or ionized amino acids. In both cases, this region may facilitate the dimerization of dimers which occurs at the catalytic site. This difference leads to a different topological arrangement of the various domains, but quite comparable final 3D structure.

The dimerization of the monomers is mainly due to the end of the second domain, and the result is a confinement of the α-helices of the two monomers in a cavity formed by β_{13} and β_{16} sheets (following the *E. Coli* numbering scheme). This edifice is stabilized by salt bridges and hydrogen bonds, and by the C-terminal chain which "embraces" the other monomer.

4.3.5 Substrate binding

The structures described above and their sequence alignments provide evidence of a binding site for ATP: the "P-loop" pattern similar to the ATP-binding domain in well known type N ATP-pyrophosphatases. However, it is more difficult to localize the citrulline and aspartate binding sites. To resolve this issue, various complexes of ASS and substrate or substrate analog have been crystallized. For *E. coli*, complexes with citrulline and aspartate (Lemke and Howell, 2001) (PDB: 1K97), with ATP only (Lemke and Howell, 2002) (PDB: 1KP2) and with ATP and citrulline (Lemke and Howell, 2002) (PDB: 1KP3) have been described; for *T. thermophilus*, complexes with ATP (Goto et al., 2002) (PDB: 1KH2), with ATP and citrulline (Goto

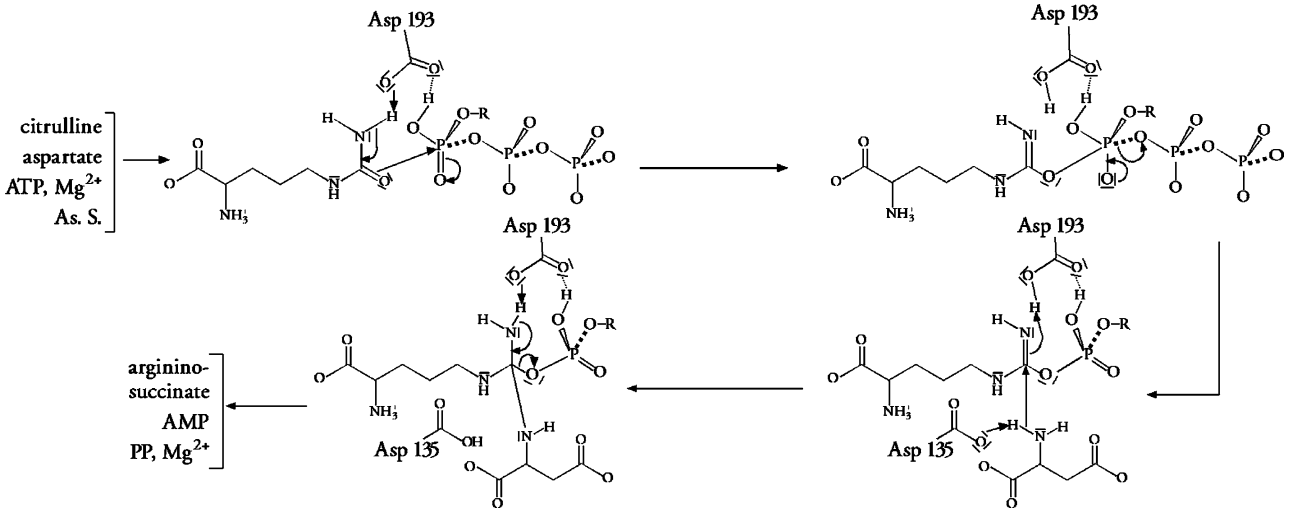

Fig. 14. Mechanism of the catalysis by ASS

et al., 2003) (PDB: 1J21), with AMP and argininosuccinate (Goto et al., 2003) (PDB: 1J20), with adenylylimidophosphate (ATP analog), arginine (citrulline analog) and succinate (aspartate analog) (Goto et al., 2002) (PDB: 1KOR), with phosphoaminophosphonic acid-adenylate ester (ATP analog), arginine and aspartate (Goto et al., 2003) (PDB: 1KH3), and with ATP, aspartate and citrulline (Goto et al., 2003) (PDB: 1J1Z) have been studied. These structures allowed to characterize the amino acids responsible for the binding of each of the substrates (cf. Figs. 13 and 14), and their spatial arrangement, thereby providing insight into the mechanism.

4.3.6 Mechanism of the reaction

A mechanism for this reaction can be proposed based on available studies (Cheung et al., 1989; Goto et al., 2002). This mechanism is summarized in Fig. 14. Kinetic studies suggest that ATP is the first substrate to be bound, followed by citrulline and then aspartate (Raushel and Seiglie, 1983), the fixation of aspartate activating the formation of the citrulline-adenylate intermediate (Ghose and Raushel, 1985). When the reaction is completed, argininosuccinate is released first, followed by pyrophosphate, and lastly AMP (Raushel and Seiglie, 1983). It has been shown (Raushel and Seiglie, 1983) that Mg^{2+} is required for the reaction; however only one crystal structure (1KH3) presents this cation. In this structure, the cation is very close to the ATP analog and the aspartate, which is consistent with its simultaneous fixation with ATP. This suggests that Mg^{2+} activates ATP by complexing its phosphate groups, enhancing their electrophilicity and lability, which is coherent with its release complexed with the pyrophosphate (Raushel and Seiglie, 1983).

However, there is an important difference between the two structural models: whereas, in *T. thermophilus* ASS, the three substrates are close enough to interact (Goto et al., 2002; Goto et al., 2003), this is not the case in *E. coli* ASS, where an important conformational change is expected (Lemke and Howell, 2002). This difference is probably related to the different nature of the intercalated domain (one helix and one "hair pin" in *E. coli*, two helices in *T. thermophilus*), which is 4 amino acids longer in type 2 enzymes, since this domain is close to the substrate binding domain. Since, like the *T. thermophilus* enzyme, the human enzyme belongs to the type 1 family, it is likely that no conformational change is needed, but this has not yet been demonstrated.

Studies by Cheung et al. (1989) suggest that urea cycle enzymes are associated in macromolecular systems to channel the substrates throughout the pathway, and Demarquoy et al. (1994) have shown that ASS can be bound on the mitochondrial membrane in variable amounts, particularly with regulation by glucagon. However, neither the protein region that may interact with other proteins, nor the region involved in the non-specific interaction with the mitochondrial membrane has been identified.

5 Citrulline transport

5.1 Citrulline transport into cells

To date, no citrulline-specific transporter has been evidenced in any cell type. However, various cell types are

Table 7. Characteristics of citrulline transport across cellular membranes. v_{max} values are for 1 mg of protein, except when marked with a star where values are for 1 mg of wet tissue

Cell type	Organism	K_m (mM)	v_{max} (nmol · min^{-1})	Reference
Aortic endothelial cell	Ox			Hilderman et al. (2000)
Aortic smooth muscle cell	Rat	1.6 ± 0.2	5.9 ± 0.6	Wileman et al. (2003)
Astroglial cell	Rat	1.1 ± 0.1	14.7 ± 0.9	Schmidlin et al. (2000)
Enterocyte	Rat	4.1 ± 0.9	$0.62 \pm 0.06^*$	Vadgama and Evered (1992)
Glial cells	Rat	3.4 ± 0.2	25.6 ± 1.4	Schmidlin et al. (2000)
Macrophage	Mouse	0.16	15–35	Baydoun et al. (1994)
	Rat	0.4 ± 0.1	26.5 ± 8.7	Schmidlin et al. (2000)
Microglial cell	Rat	1.2 ± 0.4	31.0 ± 0.2	Schmidlin et al. (2000)

able to take up or release citrulline, and several studies have demonstrated that citrulline can be transported by the usual, generic amino acid transporters; a collection of kinetic parameters for citrulline transport in various cell types is given in Table 7.

All cells in the nervous system (microglial cells, astrocytes and neurons) have a citrulline transport system (Schmidlin et al., 2000). The mechanism of this transport is not yet known, but it may use the L amino acid carrier system for long chain amino acids, since it is not sodium-dependent (Schmidlin et al., 2000).

The absorption of citrulline by endothelial aortic cells proceeds through a transport system than can also carry arginine (Hilderman et al., 2000). This suggests a different transport system to the usual y^+ system for arginine, since the y^+ system does not carry citrulline.

In rat aortic smooth muscle cells, L-citrulline transport appears to be partially Na^+-dependent and pH-insensitive. The transporter is distinct from those used by arginine. In addition, this transport system appears unresponsive to catabolic stimuli such as lipopolysaccharides and interferon γ (Wileman et al., 2003).

In macrophages, it appears that two transport systems coexist (Baydoun et al., 1994). One is a saturable system for neutral amino acid transport, while the other presents a competitive inhibition of arginine transport by citrulline ($K_i = 3.4$ mM). A transport process has also been evidenced in Jurkat cells (T lymphocytes) (Bansal et al., 2004), but its characteristics have not yet been established.

Uptake by enterocytes appears to require sodium (Vadgama and Evered, 1992). Since the system A appears to lack the apical side of enterocytes (Ray et al., 2002) and system ASC preferentially transports short chain amino acids, this suggests a transporter from family B^0.

No citrulline carrier in the kidney has yet been identified, but competition from citrulline with dibasic amino acids for transport, as suggested by citrulline accumulation in urine following addition of a lysine load (either orally or by venous injection) (Oyanagi et al., 1981), suggests its existence.

5.2 Intracellular exchanges of citrulline

As described above, citrulline is synthesized by OCT in the mitochondria but catabolized by ASS in the cytosol. Thus, a transport mechanism must exist in cells that express both enzymes, especially in cells performing a complete or partial urea cycle (Porter, 2000). This transport is performed by the ornithine-citrulline exchanger, which is localized in the inner membrane of the mitochondria. It involves, in addition to the exchanged amino acids, a proton and a Ca^{2+} ion.

6 Citrulline metabolism in mammals

Figure 15 provides a summary of citrulline metabolism in mammals. If we exclude the minor contributions of citrullinated protein degradation and dimethylarginine degradation to citrulline fluxes, the metabolism of free citrulline can be classified into three pathways that occur at different levels. The first metabolic pathway is arginine biosynthesis, which involves citrulline exchanges at whole body level; the second pathway is the NO cycle, which can involve local recycling of citrulline; the third pathway is the complete urea cycle, taking place in the liver.

6.1 Citrulline fluxes in the organism

6.1.1 Citrulline absorption and synthesis in the small intestine

Citrulline contained in food is absorbed by the intestine. Some studies have shown that, when present on the luminal side, citrulline accumulates in enterocytes, which suggests the action of a transporter (Vadgama and Evered, 1992),

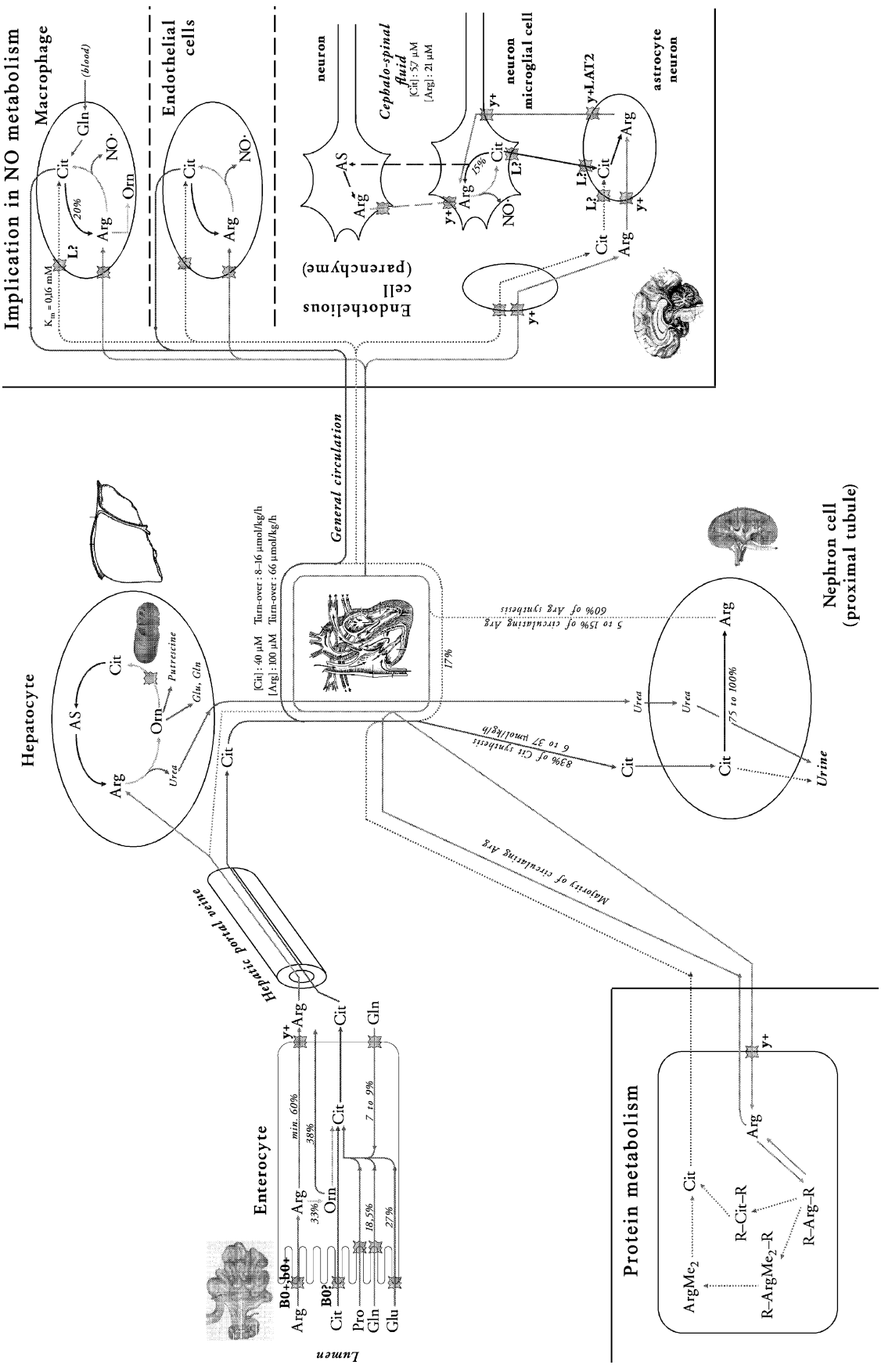

Fig. 15. Simplified metabolism of citrulline in the human adult. Numerical values, when present, correspond to values in humans. Arginine to ornithine conversion is always accompanied by urea formation, even when not represented; the conversion of citrulline to arginine is always made *via* argininosuccinate, even when omitted for clarity. *Arg*: arginine; *AS*: argininosuccinate; *Cit*: citrulline; *Gln*: glutamine; *Glu*: glutamate; *Orn*: ornithine; *Pro*: proline. (Castillo et al., 1993; Cynober, 2002; Cynober et al., 1995; Morris, 2000; Rabier and Kamoun, 1995; Vadgama and Evered, 1992; van de Poll et al., 2004; Wiesinger, 2001; Windmueller and Spaeth, 1981; Wu, 1998; Yu et al., 1996)

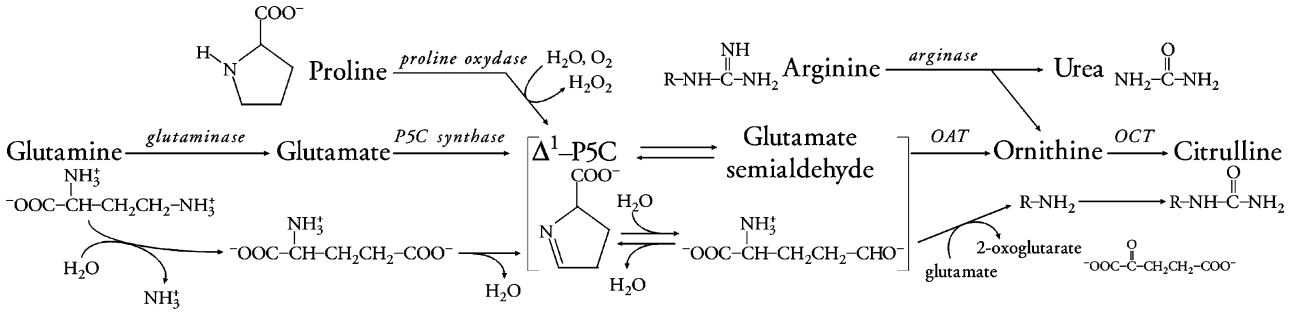

Fig. 16. Metabolic pathway of citrulline synthesis from other amino acids in enterocytes and (from glutamate) other cells. *OAT*, ornithine aminotransferase. Δ^1-P5C is in equilibrium with glutamate semialdehyde, the former being more stable for entropic reasons. The conversion is thus spontaneous and each form is available for further reactions. See text for other details

as described above. This absorption seems to be optimal between the median and lower ileum (Vadgama and Evered, 1992). However, citrulline is not an essential amino acid in physiological conditions (if we accept the extension of this notion to non-protein amino acids): most of the circulating citrulline comes from glutamine conversion in enterocytes (Wu, 1998) following the reactions described in Fig. 16. Other amino acids can also act as citrulline precursors: glutamate, proline, arginine under some circumstances (such as a hypoproteic diet, see below (Cynober, 2002)). Glutamine may come from food supply as well as from the blood (Plauth et al., 1999): enterocytes metabolize between 25% and 33% of the arterial glutamine, 66% of luminal glutamine, and 96% of luminal glutamate (Wu, 1998), while in rats, about 28% of these metabolized amino acids are converted into citrulline. Note that two glutamate molecules are required for each synthesized citrulline molecule: one gives the carbon skeleton (*via* the Δ^1-P5C), and can be replaced by other amino acids such as proline, whereas the second gives the terminal amine (*via* a direct transamination reaction) (Fig. 16). In newborn mammals, it seems that proline is the main source of citrulline, since any inhibition of this metabolic pathway leads to citrulline leakage (Dillon et al., 1999). The necessity of this citrulline synthesis in the gut is demonstrated by the effects of intestinal resection, which makes arginine an essential amino acid (Wakabayashi et al., 1995, 1994), and by the effects of a chemical inhibition of this pathway, which leads to retarded growth (Hoogenraad et al., 1985).

6.1.2 Citrulline catabolism

If it is not used in NO metabolism (see Section 6.2), citrulline is mainly metabolized in the kidney, where it is converted into arginine by cells of the nephron proximal tubules (Levillain et al., 1990). Conversion in the kidney is achieved through a partial urea cycle involving ASS and argininosuccinate lyase (ASL). Synthesized arginine is released into the general blood circulation. In adults, the citrulline converted by the kidney is enough to provide the body's full arginine requirements. Arginine synthesized from citrulline represents 60% of the de novo arginine synthesis in the organism, but only 5 to 15% of circulating arginine (Morris, 2000). Approximately 83% of the citrulline released by the gut is metabolized within the kidney (Windmueller and Spaeth, 1981), and this represents only 35% of circulating citrulline (Windmueller and Spaeth, 1981).

However, in newborns, this de novo synthesis of arginine is not enough to fulfil physiological requirements, and this citrulline to arginine reaction is also observed in the intestinal mucosa. This enzymatic activity disappears with weaning. Thus, the expression of this pathway is probably related to a deficiency of arginine content in maternal milk (Le Floc'h and Seve, 2000).

6.1.3 Significance of citrulline/arginine interorgan exchanges: the arginine biosynthesis axis

The main reason for this citrulline metabolism split between two organs is related to the efficacy of the capture of arginine by the liver. In fact, without metabolic adaptation, almost all the arginine coming from food supply would be withdrawn from the portal blood by the liver, leaving only very low amounts of available arginine for other organs. Furthermore, as arginine is a positive regulator of ureagenesis (through an activation of the N-acetylglutamate synthase (Meijer et al., 1990)), other amino acids could be inappropriately overmetabolized. Citrulline is the solution to this problem: it can be seen as a masked form of arginine to bypass the liver, since the liver is unable to uptake citrulline from portal circulation (Windmueller and Spaeth, 1981); citrulline is then

converted to arginine by the kidney and released into the blood to make it available for the whole body. Hence, the conversion of citrulline into arginine is a very important actor in nitrogen homeostasis in the body: when protein intake decreases, OCT is expressed in the intestine, promoting the formation of citrulline and thereby allowing downregulation of liver ureagenesis (Cynober et al., 1995).

6.2 NO metabolism and citrulline recycling

Because citrulline is easily converted into arginine by a half urea cycle with the action of ASS and ASL, it can be used as a precursor of NO. Hence, many cell types which are able to metabolize arginine into NO are able to uptake circulating citrulline, which explains why citrulline induces certain of the NO effects; for example, it can decrease the tonicity of blood vessel muscles (Raghavan and Dikshit, 2001).

This citrulline can also be supplied by arginine conversion into NO itself, forming the so-called NO cycle. In activated macrophages, this citrulline recycling, evidenced by Wu and Brosnan (1992), accounts for up to 20% of NO produced (Murphy and Newsholme, 1998). Such recycling also exists in endothelial cells. When NO requirements are especially important, as in the activated macrophage (Murphy and Newsholme, 1998), citrulline can even be synthesized *in situ* from glutamate, through the same metabolic pathway as in the bowel (see Section 6.1.1).

However, this cannot be extended to just any cell type, for instance to vascular smooth muscle cells, where citrulline cannot replace arginine to induce NO production (Wileman et al., 2003).

The figure seems to be even more complex in the brain, since the recycling of citrulline into arginine is split between various cell types, defining a unique inter-cell-type cycle. Indeed, the brain neurones producing NO are not able to reconvert citrulline into arginine since they do not express the ASS and ASL enzymes. Hence, citrulline is released from the neurons and taken up by surrounding neural cells where return-conversion to arginine is performed. Newly formed arginine is released and taken up by neurons to form more NO (Wiesinger, 2001).

However, if uncontrolled, this cycle could lead to an excessive production of NO which could damage the surrounding cells. This risk is limited by the inhibition of ASS by NO (Hao et al., 2004) and by the fact that arginine availability is dependent upon type II arginase activity in most cells.

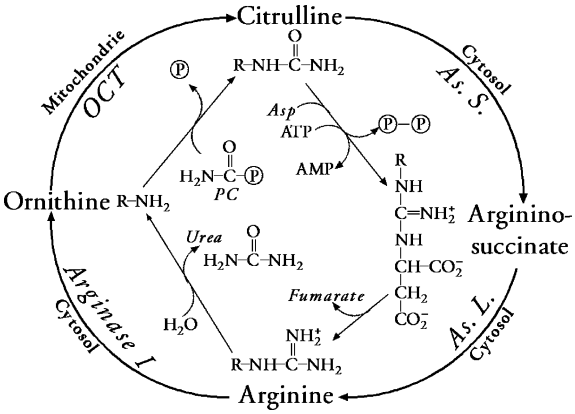

Fig. 17. The urea cycle

6.3 Liver metabolism of citrulline

Citrulline is one of the intermediates of the urea cycle in hepatocytes (Fig. 17), and can be detected as such. However, this citrulline pool is particularly labile: all synthesized citrulline is converted in argininosuccinate by cytoplasmic ASS, and there is no citrulline release into the general circulation (Windmueller and Spaeth, 1981). Furthermore, it has been shown that hepatocytes involved in the urea cycle are unable to take up citrulline from the portal circulation, and that uptake from arterial circulation is minimal (Windmueller and Spaeth, 1981). Therefore, citrulline metabolism in the liver is a strictly compartmentalized metabolism, unconnected with the other metabolic pathways involving citrulline.

7 Citrulline in medicine: from physiopathology to therapeutic agent

7.1 Inherited diseases related to citrulline metabolism

As described above, citrulline metabolism is mainly driven by three enzymes: ASS, NOS and OCT. In addition, the ornithine/citrulline mitochondrial exchanger has to be factored in. Each non-lethal mutation of the gene coding for any of these proteins can lead to inherited diseases that will be characterized by a deficient citrulline metabolism. This report will give a brief description of these diseases. All share a common consequence: since citrulline metabolism is deficient, the urea cycle does not function properly, leading to increased ammonia concentrations, followed by coma and ultimately death. Mutations of NOS are either lethal or else not directly related to diseases but ''only'' to changes in vulnerability to disease (OMIM, 2000) and they will not be dealt with here.

7.1.1 OCT deficiency

If OCT is not functional, citrulline cannot be synthesized. This will lead to increased plasma glutamine (since it is the natural precursor of citrulline), lysine and serine concentrations; conversely, citrulline concentrations in the blood will be much lower than normal, since citrulline cannot be synthesized in the gut.

This disease (OMIM code: 311250) is relatively uncommon (1 for 80 000 births) and may be dominant or recessive, depending on the involved mutation. Effects can appear at birth or very late in life. Since the OCT gene is on the X chromosome (Lindgren et al., 1984), the effects are stronger for men and are of various intensity in women.

7.1.2 Citrullinaemia

This disease is defined by an accumulation of citrulline in the body because citrulline degradation cannot take place. Three forms of the disease have been described according to the origin of the dysfunction. An increase in blood orotic acid, glutamine and alanine concentrations is also observed, accompanied by a decrease in blood arginine concentrations. This disease is mainly observed in Asian populations, especially in Japan.

Type I citrullinaemia (OMIM: 215700) is the most frequent and the most severe. It is due to a recessive mutation of the ASS gene (OMIM, 2000) on chromosome 9 (Carritt et al., 1977), which changes the kinetics of the enzyme.

Type II citrullinaemia (OMIM: 603471 for the adult variant, 605814 for newborns) is less severe and relatively infrequent (1 case for 100 000 births in Japan). Type II citrullinaemia is due to a mutation of a gene on chromosome 7 (region 7q21.3) (Kobayashi et al., 1999). This gene encodes for citrine, a protein recently identified as an aspartate-glutamate transporter, with the intervention of H^+ and Ca^{2+} (Palmieri et al., 2001). After mutation or deletion, the transport becomes inefficient and ASS cannot work because of the lack of aspartate. Interestingly, it seems that ASS is also deficient, by an unknown mechanism. The difference between newborn and adult forms seems to be unrelated to the citrine gene mutation.

Type III citrullinaemia is very rare; it is due to a major mutation of the ASS gene that prevents expression (Kobayashi et al., 1986).

7.1.3 Triple H syndrome

The disease is characterized by an increase in plasma ornithine and ammonia concentrations, and by the presence of homocitrulline in urine: *hy*perornithinaemia, *hy*perammonaemia and *ho*mocitrullinaemia. Here again, the high blood ammonia concentrations are followed by coma and ultimately death.

This very infrequent disease is due to a dysfunction of the mitochondrial ornithine-citrulline exchanger (Camacho et al., 1999), related to chromosome 13.

7.2 Citrulline as a marker of tissue function

As discussed above, the metabolism of free citrulline is mainly divided between the intestine (synthesis) and the kidney (degradation). Because of this specificity, citrulline can act as a reliable functional marker for these two organs.

7.2.1 Citrulline, a powerful marker of bowel function

Since the short bowel is the main source of circulating citrulline, it seems natural to use citrullinaemia as a marker of the small bowel function. First clinical evidence of the practicability of this idea was obtained by Crenn et al. (2000) who showed that citrulline is an efficient marker of the active small bowel mass. Since this pioneering study, other reports have established the utility of this marker in various contexts where small bowel activity needs to be monitored: bowel surgery, to control the correct implantation of transplants (Gondolesi et al., 2002, 2004; Pappas et al., 2002); bowel pathologies, such as the villous atrophy-associated small bowel disease (Crenn et al., 2003); radiation-induced intestinal epithelial damage (Lutgens et al., 2003, 2004b) or other myeloablative therapy (Blijlevens et al., 2004). Interestingly, Lutgens et al. (2004a) recently pointed out that citrullinaemia was more sensitive and more specific than the sugar-based permeability test for detecting chemotherapy-induced gut damages in patients suffering haematological malignancies.

Recent studies suggest that the correlation between plasma citrulline levels and intestinal function may even be used in a predictive manner, in particular to predict the risk of acute rejection of small bowel transplants (Pappas et al., 2004).

7.2.2 Citrulline, a potential marker of renal function

It has already been shown that citrullinaemia is a good marker of kidney function, especially of the proximal tubules, in rats. Indeed, severe renal failure is characterized

by hypercitrullinaemia (Ceballos et al., 1990), which appears even more sensitive to kidney dysfunction than the classical creatininaemia (Levillain et al., 1997).

7.3 Citrulline as a therapeutic agent

In recent decades, little attention has been given to the use of citrulline as therapeutic agent. However, the metabolism of citrulline is evidence that it is a natural precursor of arginine, which is involved in three metabolic pathways. Hence, for each of these pathways, citrulline is a putative candidate for new treatments.

7.3.1 Alternative to arginine supplementation

As explained in Section 6.1.3, citrulline can be seen as a masked form of arginine to bypass the liver. Furthermore, the fact that arginine is taken up and largely metabolized by the liver to yield urea raises questions about the safety of arginine supplementation. As described above, arginine is liable to cause excessive ureagenesis, since it acts as a catalyst of ureagenesis (Shigesada and Tatibana, 1971; Shigesada and Tatibana, 1978). Hence, citrulline is a good alternative candidate for supplementation.

The ability of citrulline to restore blood arginine levels was first reported by Hartman et al. (1994), and this idea is now explored in several ways. More generally, experimental studies have recently pointed out that citrulline could be a promising pharmaconutrient candidate to sustain protein metabolism in intestinal diseases. These studies have demonstrated the significant benefits of a citrulline-enriched diet in short bowel syndrome and in protein-energy malnutrition (PEM) in old rats.

7.3.1.1 Short bowel syndrome. In massive intestinal resection, the main site of citrulline production is greatly reduced. As a consequence of the diminished plasma citrulline levels, decreased plasma arginine is also observed (Crenn et al., 2000; Wakabayashi et al., 1994), suggesting that arginine becomes an essential amino acid after massive intestinal resection (Wakabayashi et al., 1994). On the other hand, citrulline should be a good candidate for generating arginine and improving nutritional status in massive intestinal resection. This hypothesis was largely demonstrated by the work of Osowska et al. (2004): in a model of massive intestinal resection (80% of the small intestine), we demonstrated that a citrulline-enriched enteral nutrition (1g/kg/day) was able to generate large amounts of arginine in various tissues, and totally restored nitrogen balance. It is tempting to speculate as to the possibility that citrulline, while not a component amino acid of proteins, may influence protein synthesis and become an essential amino acid in intestinal resection.

7.3.1.2 Protein-energy malnutrition in ageing. Ageing is associated with an inevitable, involuntary loss of muscle mass, a process described as sarcopenia (Roubenoff and Castaneda, 2001). Sarcopenia results in muscle weakness leading to an increased propensity for falls, greater morbidity and loss of functional autonomy (Carmeli et al., 2000; Vandervoort and Symons, 2001). There have been several proposals as to the underlying biochemical mechanisms for age-related sarcopenia (Nair, 1995, 2000; Richmonds et al., 1999; Tsao et al., 1996; Viner et al., 1999; Wanagat et al., 2001). Malnutrition is considered as one of the general causes of the slow-down of protein synthesis and turnover observed in sarcopenia (Carmeli et al., 2002). Several studies have attempted to explain this phenomenon. There is a significant increase in amino acid metabolism in the splanchnic area in elderly subjects compared to adults subjects (Boirie et al., 1997). This higher splanchnic extraction in the elderly leads to inadequate systemic plasma level of amino acids in the postprandial period (Mosoni et al., 1995) which may explain resistance to renutrition in malnourished elderly populations (Hebuterne et al., 1995). Since hyperaminoacidaemia (together with insulin) is one of the main stimulating factors for protein synthesis (Pacy et al., 1994), the insufficient increase in postprandial plasma amino acid concentrations should result in a blunted protein synthesis rate. With the use of an amino acid which escapes splanchnic extraction, it would be possible to deliver more adequate amounts of nitrogen to the peripheral tissues, including muscles, and thus to increase protein synthesis. Citrulline, which fulfils these criteria, is therefore a good candidate. In fact, this hypothesis was confirmed recently by Duchemann et al. (2004) who observed that citrulline supplementation in malnourished aged rats increases muscle protein content by stimulating protein synthesis.

7.3.2 Towards a control of NO metabolism?

More and more studies have shown that citrulline can act as an arginine precursor for the NO cycle (see Section 6.2), not only because of the renal conversion but also because of local *in situ* conversion *via* ASS and ASL (Section 6.2). Hence, citrulline administration may constitute a therapeutic strategy for controlling NO metabolism disorders. Several studies have been conducted based on

this idea, giving such encouraging results that a patent protecting this idea has already been filed (Waugh, 1998).

7.3.2.1 Immunostimulation. Arginine becomes essential in stress conditions. One explanation for this is the significant degradation of arginine into urea by arginase, leading to a decrease in immunological status (Bansal et al., 2004). Recent research suggests that immunological status can be improved in stress situations by providing citrulline to T-lymphocytes, which can use it to generate the arginine they need *in situ* (Bansal et al., 2004).

NO also plays an important role in the defence against invading organisms. Stimulating NO production by macrophages should therefore be a good way to enhance protection against infections. Studies have been performed along these lines, first by Norris et al. (1995) against parasitosis by *Trypanosoma cruzi*, and more recently by Zheng and Lin (1998) for parasitosis by *Toxoplasma gondii*. Both studies suggest that citrulline is an efficient source of NO for macrophages in these challenging situations.

7.3.2.2 Blood pressure control. An initial study showed that citrulline malate may have an effect on dystonia associated with arterial hypotension (Oknin et al., 1999), which may be related to citrulline being involved in NO biosynthesis for arterial blood pressure control.

Complications can occur in sickle cell disease because the distorted haematocytes do not circulate well in blood vessels. Vasodilation and vasoprotection therefore become especially important. Since these processes are enhanced by NO, any substrate that can produce NO may improve the patient's condition. Based on these considerations, Waugh et al. (2001) performed a study with oral citrulline as an NO precursor *via* arginine. The results of this phase II clinical trial are very encouraging, showing a clear improvement in patient outcome with no reported side effects.

7.3.3 Citrulline as a hypoammonaemic agent

Since citrulline is an intermediate of the urea cycle, it seems logical to use it as a way to trap excess ammonia, and thus decrease ammonaemia. However, since citrulline is not taken up by the liver, a direct effect of citrulline cannot be expected. Instead, citrulline will be converted into arginine in the kidneys and this arginine will eventually be taken up by the liver, where it will be degraded into ornithine, releasing urea: the urea cycle is thus restored, but thanks to collaboration between liver and kidney. This means citrulline can only act as an efficient hypoammonaemic agent if the citrulline to arginine pathway is efficient, which excludes ASL and ASS dysfunction-related pathologies and renal failure, which can complicate liver failure.

7.3.3.1 Permanent hyperammonaemia. The main class of disorders that cause hyperammonaemia includes the various pathologies of the urea cycle. Citrulline is given as an efficient hypoammonaemic agent in OCT deficiency, carbamoyl phosphate transferase (CPT) deficiency (CPT is the enzyme that synthesizes carbamoyl phosphate, one of the OCT substrates in the urea cycle) and triple-H syndrome (Awrich et al., 1975): in all these pathologies, the ASS and ASL enzymes remain unaffected.

Some other inherited pathologies can also lead to hyperammonaemia. For instance, in lysinuric protein intolerance (dibasic aminoaciduria type 2), hyperammonaemia is caused by a lack of arginine, which is not absorbed by the enterocytes because of a dysfunction of the basal side cationic amino acid transporter. However, since citrulline is neutral, it uses another transporter and it can be absorbed to provide the necessary arginine, and is therefore an efficient treatment in this situation (Rajantie et al., 1980; Mizutani et al., 1984).

7.3.3.2 Transient hyperammonaemia. Hyperammonaemia can also occur as a treatment side effect. For instance, when treating epilepsy with valproate, encephalopathy is a frequent side effect due to the increase in plasma ammonia levels. It has been shown, at least in the rat, that the administration of citrulline lowers the risk of this side effect (Stephens and Levy, 1994).

A special case of temporary hyperammonaemia is muscular effort, where it contributes to the feeling of fatigue. Among the various antiasthenia treatments available, citrulline malate (Stimol® from Biocodex laboratories) acts on this hyperammonaemia to give a quicker lowering of ammonia levels (Callis et al., 1991; Vanuxem et al., 1990). It also acts on lactate metabolism (Fornaris et al., 1984) and enhances the aerobic production of ATP in the muscle (Janeira et al., 1998), but this latter effect seems to be related to malate and not to citrulline (Bendahan et al., 2002).

8 Citrullinated proteins

Citrulline is involved in post-translational modifications of certain proteins. This fact has been known for a long time now, but only recently has more attention been paid

to this field since recent reports suggested a relationship between these post-translational modifications and numerous pathologies as well as a growing number of biological functions.

8.1 Origin

As mentioned previously, there is no codon for citrulline; hence, any citrulline found in a protein sequence must come from a post-translational modification of the protein. This citrulline pool is therefore (almost) completely independent of the free citrulline pathways. This modification arises on arginyl residues, which are deiminated by a specific enzyme, protein-arginyl deiminase (PAD, EC 3.5.3.15), according to the following reaction:

$$\text{Arginyl} + H_2O \longrightarrow \text{Citrullyl} + NH_4^+$$

Five isotypes of PAD, numbered I to V, have been identified to date. Only four appear to be present in humans (I, II, III and V); four also are present (I to IV) in mice and other rodents. PAD seems to be Ca^{2+}-dependant and, *in vitro*, may also act on free arginine (with $K_m = 33.4$ mM, for the murine enzyme).

Since this enzyme is present in various tissues and is active on many proteins, the biological role of the deimination may be important, but is not well understood. The following is a summary of current knowledge in this intriguing field.

8.2 Role in keratinization

8.2.1 Biological role

The best known role of PAD is the modification of the proteins involved in epidermal keratinization, especially in skin appendages. Main targets are trichohyalin, keratin and filaggrin.

PAD type II modifies filaggrin and K1 keratin in the *stratum corneum* of the epidermis (Ishigami et al., 2002). The role of these modifications is not clearly established. Filaggrin is an aggregative protein for epidermal keratins. It is synthesized as a protein oligomer (the profilaggrin) which forms keratohyalin granules. After dephosphorylation, profilaggrin is cleaved by proteases, then transformed under the action of PAD. It is possible that the modification of charge due to the arginyl to citrullyl conversion (since arginine is cationic and citrulline neutral) changes the interaction with keratin, allowing modified filaggrin to aggregate keratins by salt bridge formation. In hairs, trichohyalin is modified by isotype III of the PAD, which in humans is found in the hair medulla and in the capillary follicle of the hair root sheath (Kanno et al., 2000). A similar mechanism may be involved in growing cells in the medulla, since trichohyalin is also synthesized in granules that are then transformed into filaments similar to intermediate filaments (Rogers et al., 1997).

8.2.2 Related pathologies

The citrullination dysfunction seems to be related to psoriasis, an epidermic disease that induces abnormally fast epidermis turnover associated with itching and inflammations, and which can affect up to 10% of the total skin surface. The citrullination of proteins is much lower in the affected epidermic areas than in healthy areas, and the citrullinated keratin is almost missing (Ishida-Yamamoto et al., 2000).

8.3 Implication in the nervous system

8.3.1 Biological role

One of the main proteic components of myelin is the basic myelin protein (up to 35% in the central nervous system). There are various isoforms of this protein, and each form presents diverse ''charge isomers'', that differ by a more or less complete conversion of arginine into citrulline. This transformation is catalyzed by the type II PAD mainly present in microglial cells.

The exact role of this modification is not yet understood. Some experiments suggest that rendering the protein apolar increases the aggregation (in the presence of lipophilin) but decreases the interaction with anionic phospholipids (Ishiyama et al., 2001; Wood and Moscarello, 1989). Therefore, it may also be a way of modulating the properties of the protein by modification of its net charge. However, while modified proteins are found in ''normal'' myelin, it is not clear whether this modification is required (and, if so, in what proportion) or only accidental, leading to the degradation of the non-functional protein.

8.3.2 Related pathologies

Multiple sclerosis, which also presents characteristics of auto-immune diseases, may be related to citrullination dysfunctions of the myelin basic proteins (Moscarello et al., 2002; Nicholas et al., 2004). Since changes in citrullination alter protein characteristics, an abnormal citrullination can have two effects: first, the changes may modify the interaction with lipids, which may cause a ''direct'' degradation of myelin, as observed in sclerosis.

Secondly, they may reveal antigenic sites recognized by plasma antibodies, which may explain the auto-immune aspect of the disease.

8.4 Other potential implications

8.4.1 Biological roles

The presence of PAD has been evidenced in other types of cells, which suggests an implication in a number of biological pathways.

The type V PAD has been evidenced in some leukocyte nuclei when it differentiates into granulocyte or monocyte (Nakashima et al., 1999), suggesting an implication in the differentiation process (given that PAD is present only at that precise step of myeloid differentiation). Suggested targets are B23 nucleophosmin and some histones (H3, H2A and H4) that may contain up to 10% citrulline (Hagiwara et al., 2002).

Some mouse studies have evidenced the presence of type I PAD in the female genital system (cytosol of epithelial cells in the uterus lumen), with enzyme concentration varying not only with female sexual hormones but also within the gestation period (lowering at the start of pregnancy, peaking in the middle) (Arai et al., 1995). The exact role of this PAD remains unknown.

8.4.2 Related pathologies

Several studies have demonstrated that, in rheumatoid arthritis, the serum contains antibodies targeted toward citrullinated peptides which wrongly attack some citrullinated proteins from articular cells. Citrullinated filaggrin can therefore be used as a diagnostic marker of this disease.

However, the origin of these citrullinated proteins is not fully understood. The available data suggest an abnormal production of PAD in the joints: for unknown reasons, some cells may undergo incomplete apoptosis, delivering some PAD (which is suspected to modify the vimentin protein during apoptosis). PAD overactivity may then attack the cytoskeleton proteins (which are similar to filaggrin) in the articular cells, revealing epitopes recognized by the circulating antibodies (Asaga et al., 1998; Girbal-Neuhauser et al., 1999). The destruction of the targeted cells is the reason behind the disease symptoms.

9 Conclusion

Citrulline, because it is not used in protein synthesis, in contrast to classical amino acids, was a long time considered only as a metabolic intermediate, especially in the context of the urea cycle. However, with extensive research performed over the past decade, citrulline appears to play a much more important role than previously thought. As emphasized in this review article, it seems to play a considerable role in metabolism and the regulation of NO.

These data highlight the importance of citrulline as a potential therapeutic agent in various pathologies; one of the more promising strategies is almost certainly through its interaction with NO metabolism. It also appears that citrulline could be a promising, safe and cheap therapeutic tool for short-bowel syndrome patients and in the treatment of sarcopenia. However, there are still obscure parts of the citrulline metabolism that should be resolved before these goals can be achieved. One of the points that is the least well understood is the mechanism of citrulline transport into cells: while the description of citrulline metabolism involves numerous between-cell or cell-blood exchanges, very little is known of the transporters involved. More generally, the control of the fluxes of citrulline between different metabolic pathways is not yet clearly understood. A better understanding of all these points is a challenge that may have wide applications in developing the therapeutic usage of this interesting amino acid.

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