Unprecedented Transformation of Aspartyl Peptides by Conjugative Degradation

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Simultaneous covalent conjugation and cleavage of aspartyl peptides observed in model experiments may give an explanation of some aggregative phenomena in defective metabolism as well as of decomposition during processing and storage of peptides and proteins.

Aspartic acid (Asp) and asparagine (Asn) are source of numerous undesired transformations in peptides.[1] Their presence increases the danger of deamidation, cyclization, transpeptidation and racemization throughout the synthesis and purification of peptides, and during lyophilization and storage of incorrect choice. The great challenge of synthesizing, purifying, and isolating of peptides is to counterbalance their extreme sensitivity to cyclization, isomerization and racemization by Asp and Asn residues.[2] The high reactivity of these residues may play a very significant role in the behaviour of oligopeptides and proteins under the physiological conditions of metabolism,[3–6] (in)activation[7–12] as well as in the aging processes[13,14] and diseases.[15,16]

Here we report a novel reaction route of the various potential transformations in aspartyl peptides, which may raise as many new problems as it solves. Arg-Lys-Asp-Val [thymopoietin(32-35), thymocartin, **1**] is an immunomodulating drug candidate.[17,18] Preliminary stability studies of tetrapeptide **1**, as expected, resulted in partial formation (10%) of aminosuccinyl (Asu) and transpeptidated isoaspartyl as well as shorter linear peptides and cyclic dipeptides. Accelerated stability studies of **1** in a concentrated solution in water or in dilute acetic acid at elevated temperature led to partial formation of very polar products (10%), that were undetected earlier on TLC. At room temp. prolonged reaction time was needed, while in dilute solutions only traces of these products could be detected. Having studied side reactions we always attempted to drive them to completion,[19] however, the approach failed in this case owing to the usual instability of peptides under hydrolytic conditions.

Two novel decomposition products were isolated with a combination of usual column chromatography on silica gel and reversed-phase HPLC. Homogeneity of the products was proved by TLC and analytical reverse-phase HPLC. Structure elucidation by amino acid analysis, and FAB–MS revealed a heptapeptide composition of Arg 2, Lys 2, Asp 2 and Val 1, and a molecular mass 917.

Uncertainties in assigning the location of the newly formed peptide bond(s) prompted us to synthesize model peptides with the usual methods in solution from the corresponding monomer units, provided with an appropriate protecting group combination.[20] The segment condensation methods we used may have caused a partial epimerization at the activated C-terminal residues, and the chiral integrity of the compounds was not confirmed. The following heptapeptides were prepared: Arg-Lys-Asp-Arg-Lys-Asp-Val **2**, Arg-Lys-iAsp-Arg-Lys-Asp-Val **3**, Arg-Lys-Asp(Val)-Arg-Lys-Asp **4**, Arg-Lys-Asp(Arg-Lys-Asp)-Val **5**, Arg-Lys(Arg-Lys-Asp)-Asp-Val **6** and Arg-Lys(Arg-Lys-iAsp)-Asp-Val **7**. All these synthetic heptapeptides as well as the isolated decomposition products were characterized by TLC and HPLC, ^{1}H NMR spectra and some of them by partial, manual Edman degradation. In addition to HPLC studies, unequivocal identification of the decomposition products with normal **2** and isoheptapeptide **3** was accomplished by ^{1}H NMR studies.

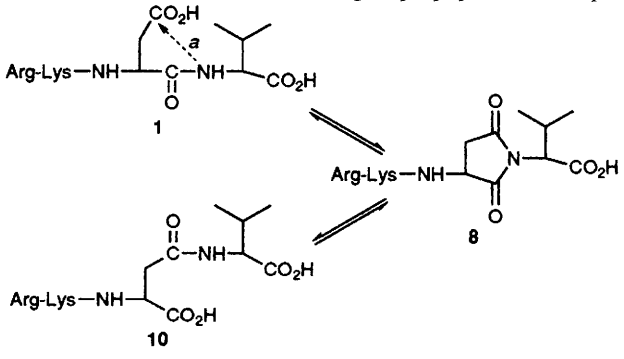

Fig. 1 Intramolecular nucleophilic substitution of **1** results in the cyclization to **8**, the hydrolysis of which leads as a result of ring opening to **1** and **10**

The optimal pH range of the formation of the novel by-products was 4 to 6. At pH 6 (60 °C, 40 h, c = 10% mass to volume) HPLC study of the reaction mixture revealed the presence of **2**, **3** (5% each), Arg-Lys-Asu-Val (**8**, 2–3%), and Arg-Lys-Asp (**9**, ~6%). In a more acidic milieu, less imide **8** (~1%) was detected in favour of a significant amount of transpeptidated isopeptide Arg-Lys-Asp(Val) (**10**, 10%), in addition to **2** and **3** (3% each), ~6% of **9** was formed.

Our previous experience with peptide chemical side reactions involving Asp, Asn and Asu peptides,[21,22] drew our attention to the role of the penultimate Asp residue which is very prone to transpeptidation after it is cyclized to a succinimide derivative **8**. In case **8** was an intermediate in the conjugative degradation, the exposure to the usual conditions of a mixture of **1** and **8** would yield a higher amount of heptapeptides. However, an attempted transformation of synthetic **8**[17] in the presence of a fourfold excess of **1**, under the conditions of the accelerated stability studies, failed to increase the amount of the expected mixture of heptapeptide derivatives. Really, this mechanism could lead to dimerization without providing any explanation for the observed cleavage of one of the C-terminal Val units (Fig. 1). However, we have to emphasize that parallel to the formation of **2** and **3**, a significant amount of **8** was detected in HPLC. Thus, under the conditions we studied, two different mechanisms of decomposition seems to operate: (*i*) imide formation through the attack on the CO carbon atom in the side-chain on the NH nitrogen subsequent to Asp (Fig. 1; route *a*), followed by the hydrolysis of imide **8** to **1** and **10**; (*ii*) the attack of the side-chain CO_2^- oxygen on the α-CO carbon atom of **1** (Fig. 2,

Table 1 Mass spectrometric study of conjudegradation on model peptides

Peptide[a]	MH^+, detected in the reaction mixture: At start	After exposure	M_W of novel product(s) expected
Ala-Lys-Asp-Val **13**	432	432, **746**	745
Arg-Ala-Asp-Val **14**	460	460, 442, 473, **802**	801
Arg-Lys-Ala-Val **15**	473	**437**	—
Lys-Asp-Lys-Leu-Glu **16**	632	632, **857** (?)	875

[a] 10% mass to volume solution of model peptides in 1% v/v aqueous acetic acid were kept at 60 °C for 40 h. The FAB–MS of both unexposed and exposed reaction mixtures was carried out on a KRATOS MS-80 instrument. The accelerating voltage was set to 4 kV. Ionization was accomplished by a beam of neutral Xe atoms of 8 keV energy. The magnet was scanned at a rate of 10 s dec^{-1} in a mass range of 1800 to 100. Resolution: 1800 (10% valley). Glycerol-1% TFA was used as matrix.

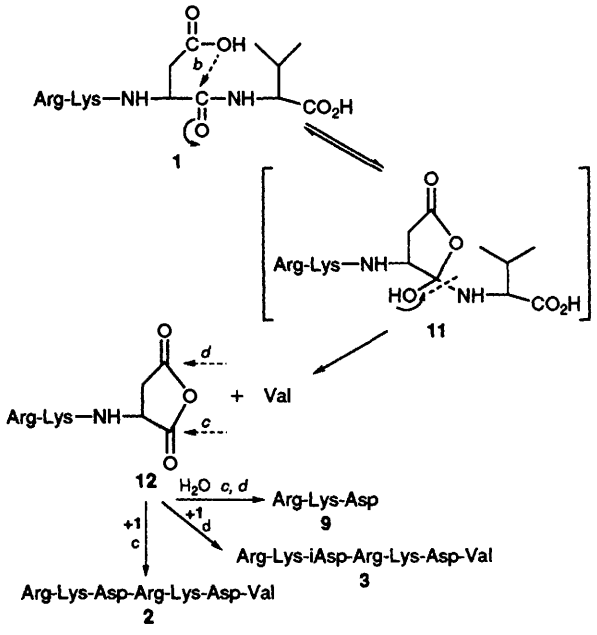

Fig. 2 A plausible explanation for conjugative degradation. It must be stressed that this mechanism operates simultaneously with imide formation (Fig. 1, route *a*).

route *b*) gives a transition state species **11**, which then partially transforms to anhydride **12** plus Val. Then the reaction of anhydride **12** with an enormous excess of the educt **1** results in **2** and **3** (Fig. 2, routes *c* and *d*, respectively). Also **12** is the source of **9** as a result of hydrolysis. Although we could not detect it we cannot exclude the possibility that dehydration of **11** leads to an isoimide derivative, which may be a stable species and may exist in equilibrium with **11** (not shown). We have previously reported competitive displacement reactions of the side-chain carboxamide group in the active esters of protected Asn and Gln derivatives.[23] In addition, the operation of an alternative, more complex mechanism must not be excluded.

The well-known sensitivity of aspartyl peptides frequently results in peptide bond cleavage, *e.g.* the Asp–Pro bond can be selectively cleaved in mild acidic milieu.[24] Fig. 2 shows the proposed mechanism of this hydrolytic decomposition *via* the intramolecular anhydride **12** of the C-terminal Asp residue, the formation of which was proposed by others.[25–27] Anhydride **12**, which we did not isolate, may be the intermediate in the formation of **2** and **3**. If the intermediacy in heptapeptide formation of this anhydride is accepted, simultaneously an explanation is given for the mode of cleavage of the residue, Val, subsequent to Asp. This is the first example of covalent intermolecular conjugation of aspartyl peptides. Simultaneous with our investigation, independent studies on the stability of insulin[7–9] have revealed a similar but intracrystal and interchain covalent 'dimerization' due to nucleophilic substitution of an N-terminal amino group on the side-chain $CONH_2$ moiety of Asn residues without any intrachain cleavage. A cardinal role was attributed to the existence in a hexamer of crystalline insulin.

In concentrated solution, the existence of a head-to-tail ion-pair, (stabilized by terminal amino and carboxyl and side-chain guanidine functions) was proposed to play a role in the dimerization process.[28] A pH dependence of dimerization and polimerization of insulin chains was proposed to be related to the existence of non-covalent dimer and hexamer molecules. To study this suggestion and the scope of the reaction, Ala-Lys-Asp-Val **13**, Arg-Ala-Asp-Val **14**, Arg-Lys-Ala-Val **15** and Lys-Asp-Lys-Leu-Glu **16** as model peptides were exposed to the same conditions. The reaction mixtures were investigated by FAB–MS so that protonated species of model peptides and dimer-like molecules could be detected directly. The results, presented in Table 1 show clearly that (*a*) conjugation is fixed to the presence of an intrachain aspartyl residue: **15** behaves like a negative control; (*b*) participation of Arg is dispensable in the reaction as in **13**. In the FAB–MS spectrum of **16** no peak at the expected molecular mass of 875 could be detected. Instead, a species of 857 appeared, indicating the loss of a water molecule, probably due to cyclization of an Asp residue to an Asu derivative.

In conclusion, this mechanism may be generalized to other peptides and even proteins and enzymes, so this conjugative degradation (conjudegradation) may be an alternative route of irreversible denaturation of proteins and (thermo)inactivation of enzymes, where only dimerization/oligomerization mediated by intermolecular disulfide interchange, has been hitherto proposed for or detected in products of higher molecular mass.

We thank Mr Attila Rill for HPLC studies, Mr Gábor Balogh for NMR studies, Dr Éva Csizér for MS spectra, Dr Gábor Dibó for manual Edman-degradation studies, Professor Dr Sándor Bajusz for valuable discussions, and Shirley Peterson (The Wistar Institute, Philadelphia) for brushing up the English of the original manuscript.

Received, 6th August 1993; Com. 3/04763F

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