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Gas-phase dissociation of hemoglobin

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Abstract

The collisionally induced gas-phase dissociation of protein assemblies of hemoglobin has been investigated. In particular, the gas-phase disassembly of the holo-hetero-dimers and the holo-tetramers of bovine, porcine, and human hemoglobin have been studied. The holo-hetero-dimer ions dissociate primarily in apo-α-chains and holo-β-chains, whereby the apo-α-chain ions accommodate more than a fair share of the initial charges present on the precursor ions. Comparing the collision-activated dissociation tandem mass spectrometry of three holo-tetramer assemblies one of the most important findings is that also these ions dissociate predominantly into the α-chain monomer concomitant with the $\alpha\beta_2$ trimer, whereby the α-chain monomer retains primarily 0 or 1, and the trimer 2–4 heme groups. Again the apo-α-chain ions accommodate more than a fair share of the initial charges, sometimes even more than the much larger $\alpha\beta_2$ trimer. The disparate charge distribution following the dissociation is in agreement with previous observations on other protein assemblies, and may to some extent be explained by assuming a charge-droplet fission model. Some significant differences are observed in the gas-phase disassembly of the hemoglobin complexes originating from the three different species. It is thought that these differences originate from differences in the gas-phase structures of these assemblies. The observed gas-phase dissociation patterns are in sharp contrast to the known solution-phase behaviour, providing a further indication that caution has to be taken when relating gas-phase data to solution-phase properties. (Int J Mass Spectrom 210/211 (2001) 637–649) © 2001 Elsevier Science B.V.

Keywords: Hemoglobin; Noncovalent complexes; CAD MS/MS; Protein assemblies

1. Introduction

Over the last decade (nano-)electrospray has allowed the relatively gentle phase transfer of biomolecules from solution to the gas phase and thus enabled the detection by mass spectrometry of larger intact multiprotein assemblies [1–13]. Complete protein assemblies, exhibiting molecular weights of over 1 mil Da, may now be transferred into the vacuum of the mass spectrometer and their assembly and disassembly may be studied inside the mass spectrometer [7,8,14–19]. Several studies have shown that mass spectrometric data may be related to solution phase features of protein assemblies, although it always should be realized that in the mass spectrometer just non-native isolated gas-phase ionic species are analyzed. Thus gas-phase collision-activated dissociation (CAD) tandem mass spectrometry (MS/MS) may sometimes be a valuable tool in the investigation of higher order structures of protein assemblies [7,10–14,18], but the obtained information should be care-

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Dedicated to Nico Nibbering with many thanks for his support and infectious enthusiasm over the years.

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fully evaluated when brought into relation with solution phase characteristics [3,20,21]. In this article we investigate in detail the gas-phase dissection by CAD MS/MS of noncovalent hemoglobin assemblies, originating from bovine, porcine, and human sources.

Hemoglobin is among the most comprehensively studied quaternary protein complexes. In vivo and in vitro at neutral pH, moderated salt concentrations and low protein concentration hemoglobin is a tetramer of the form $(\alpha H)_2(\beta H)_2$, containing four heme groups and exhibiting a molecular mass of approximately 64.5 kDa. Both the α- and β-chains bind noncovalently to a heme group, which has a molecular mass of 615 Da. It is well established that in solution tetrameric hemoglobin may undergo reversible dissociation into subunits under influence of pH, salt concentration and other experimental variables [22]. The dissociation equilibria of hemoglobin in solution are usually represented in terms of the simple scheme:

$$(\alpha H\beta H)_2 \rightleftharpoons \alpha H\beta H + \alpha H\beta H$$

$$(\alpha H\beta H) \rightleftharpoons \alpha H + \beta H$$

In acetate buffers below pH = 6.5 tetrameric hemoglobin dissociates into $\alpha H\beta H$ hetero-dimers, below pH = 5 the dissociation process even goes further than the dimer involving single chains as well. Also, at more alkaline pH values the tetramer dissociates into dimers. Hemoglobin protein assemblies have also been studied in the gas phase by electrospray ionization mass spectrometry [3,23], in fact the intact tetrameric protein complex was one of the first reported noncovalent protein complexes. To some extent even the gas-phase dissociation of hemoglobin protein assemblies has been studied, mostly by nonselective so-called nozzle-skimmer fragmentation, i.e. without precursor ion selection.

2. Experimental

Mass spectrometry measurements were performed on a quadrupole time-of-flight (Q-TOF) instrument (Micromass Ltd., Manchester, UK) operating in positive ion mode, equipped with a Z-spray ion source. Unless stated otherwise spectra were recorded from a 10 μM protein solution in aqueous ammonium acetate (50 mM) solutions at a pH of 5.9. Nanoelectrospray needles were made from borosilicate glass capillaries on a P-97 puller (Sutter Instrument Co., Novato, CA). Needles were coated with a thin gold layer (~500 Å) using an Edwards Scancoat Six sputter coater. The potential between the nanospray needle and the orifice of the mass spectrometer was between 1300 and 1500 V, the cone voltage was set to 50 V. In MS/MS mode the quadrupole was used to select the precursor ions with a mass window of approximately 10 Da. These were subsequently fragmented in the hexapole collision cell, generating product ions that were mass analyzed by the TOF mass analyzer. Unless stated otherwise for CAD MS/MS measurements the voltage over the hexapole collision cell was set to 35 V (for normal mass spectrometry spectra this is 4 V). Argon was used as acollision gas at a pressure of 2.0 bar (as indicated by the pressure readout).

Materials: Bovine, porcine, and human hemoglobin were obtained from Sigma (Zwijndrecht, The Netherlands) and used without further purification. For human hemoglobin the apo-α-chain has an average mass of 15 126.4 Da and a calculated isoelectric point (pI) of 8.7, whereas the apo-β-chain has a mass of 15 867.2 Da and a pI of 6.8. For porcine hemoglobin the apo-α-chain has a mass of 15 039.1 Da and a pI of 8.8, whereas the apo-β-chain has a mass of 16 035.4 Da and a pI of 6.8. For bovine hemoglobin the apo-α-chain has a mass of 15 053.2 Da and a pI of 8.2, whereas the apo-β-chain has a mass of 15 954.4 Da and a pI of 7.0. These masses could be confirmed by mass spectrometry.

3. Results

The normal mass spectrometry spectra of hemoglobin recorded from the aqueous ammonium acetate buffers revealed ion signals originating from the tetramer, dimer, and monomer species, and were quite similar to those reported previously [3,23]. At basic pH 8.5 an optimum was found for tetramer detection. However, at such basic pH the protein complexes

exposed many small adducts. At a pH of 5.9 a lesser amount of tetramer was observed in the mass spectra, albeit the ion signals were much narrower. Therefore, all CAD MS/MS experiment were performed on ions obtained from an 50 mM aqueous ammonium acetate solution acidified to pH = 5.9, using acetic acid. We now first discuss the CAD MS/MS data obtained for the intact αHβH hemoglobin hetero-dimers, which are subsequently compared with the data obtained for the intact noncovalent holotetramer $(\alpha H)_2(\beta H)_2$ assemblies.

3.1. CAD MS/MS of hemoglobin hetero holo-dimers

The most abundant ions observed in the mass spectra of hemoglobin electrosprayed from a ammonium acetate solution at pH = 5.9 were the intact αHβH hemoglobin hetero-dimers exhibiting between 10 and 12 charges. For the CAD MS/MS data spectra were compared of the $[(\alpha H)(\beta H)]^{11+}$ ions of the intact holo-dimer of bovine, porcine, and human hemoglobin, respectively, taken under exactly identical experimental conditions. Fig. 1 gives an overview of these CAD MS/MS spectra and at first glance they all look quite similar. Moreover, CAD MS/MS spectra of other charge states of the dimer provided similar data. In the region $2000 < m/z < 2800$ the most abundant fragment ions were relatively highly charged apo-α-chains exhibiting a charge of 8, 7, or 6 ($[\alpha]^{7+}$ being the most abundant fragment ion). Also in this region less abundant highly charged β-chain ions, e.g. $[\beta]^{7+}$, were observed. Additionally, at lower *m/z* values free singly charged heme ions ($m/z = 616$), but also heme-dimers were observed (around $m/z = 1250$), originating from $[\text{heme}]_2$ cationized by a H^+, Na^+, or K^+. In the region $3500 < m/z < 6000$ relatively low charged fragment ions were observed primarily originating from β-chains exhibiting a charge of 4 or 3 (i.e. the $[\beta]^{4+}$ and $[\beta H]^{4+}$ ions). In this *m/z* region much less abundant low charged α-chain ions, e.g. $[\alpha]^{4+}$, could be observed. Finally, around the *m/z* of the precursor ions the fragment ions $[\alpha\beta H]^{11+}$, $[\alpha\beta H]^{10+}$ and $[\alpha\beta]^{10+}$ were observed which correspond, respectively, to the loss of a neutral or singly charged heme group and the loss of a singly charged heme-dimer.

3.2. CAD MS/MS on the hemoglobin holotetramers

For the CAD MS/MS experiments on the intact noncovalent holotetramer $(\alpha H)_2(\beta H)_2$ complex the most abundant tetramer ions with 17 charges were mass-selected (i.e. the $[(\alpha H)_2(\beta H)_2]^{17+}$ ions). Fig. 2 gives an overview of the CAD MS/MS spectra of the $[(\alpha H)_2(\beta H)_2]^{17+}$ ions of, respectively, bovine, porcine, and human hemoglobin, taken under identical experimental conditions as for the dimers. The three-tetramer spectra looked again quite similar. CAD MS/MS spectra of other charge states of the tetramer provided similar data. The most abundant ions observed in the gas-phase CAD MS/MS dissociation of the $[(\alpha H)_2(\beta H)_2]^{17+}$ ions were apo-α monomer chains exhibiting a charge between 6 and 10 (with the $[\alpha]^{9+}$ ion being the most abundant). Therefore, the most important dissociation product in the dissociation of the holo-tetramer is identical to the one observed in the dissociation of the holo-dimer. Interestingly, no ion signals were observed originating from any form of a dimeric species. Additionally, at lower *m/z* values free singly charged heme ions ($m/z = 616$), but also singly charged heme-dimers were observed (around $m/z = 1250$). In the region $4500 < m/z < 7500$ relatively low charged fragment ions were observed primarily originating from $\alpha\beta_2$ trimers exhibiting a charge between 9 and 7 ($[\alpha\beta_2 H_3]^{8+}$ being the base peak in this area). Finally, around the *m/z* of the precursor ions $[\alpha_2\beta_2 H_3]^{16+}$ and $[\alpha_2\beta_2 H_2]^{16+}$ ions were observed, which corresponded to the loss of singly charged heme group or the loss of a singly charged heme-dimer, no loss of neutral heme could be detected.

4. Discussion

4.1. CAD MS/MS on the hemoglobin hetero holo-dimers

Directly obvious from the CAD MS/MS data presented in Fig. 1 is the apparent preference of the dimer to dissociate into two protein fragments, whereby one of the fragments takes up a more than

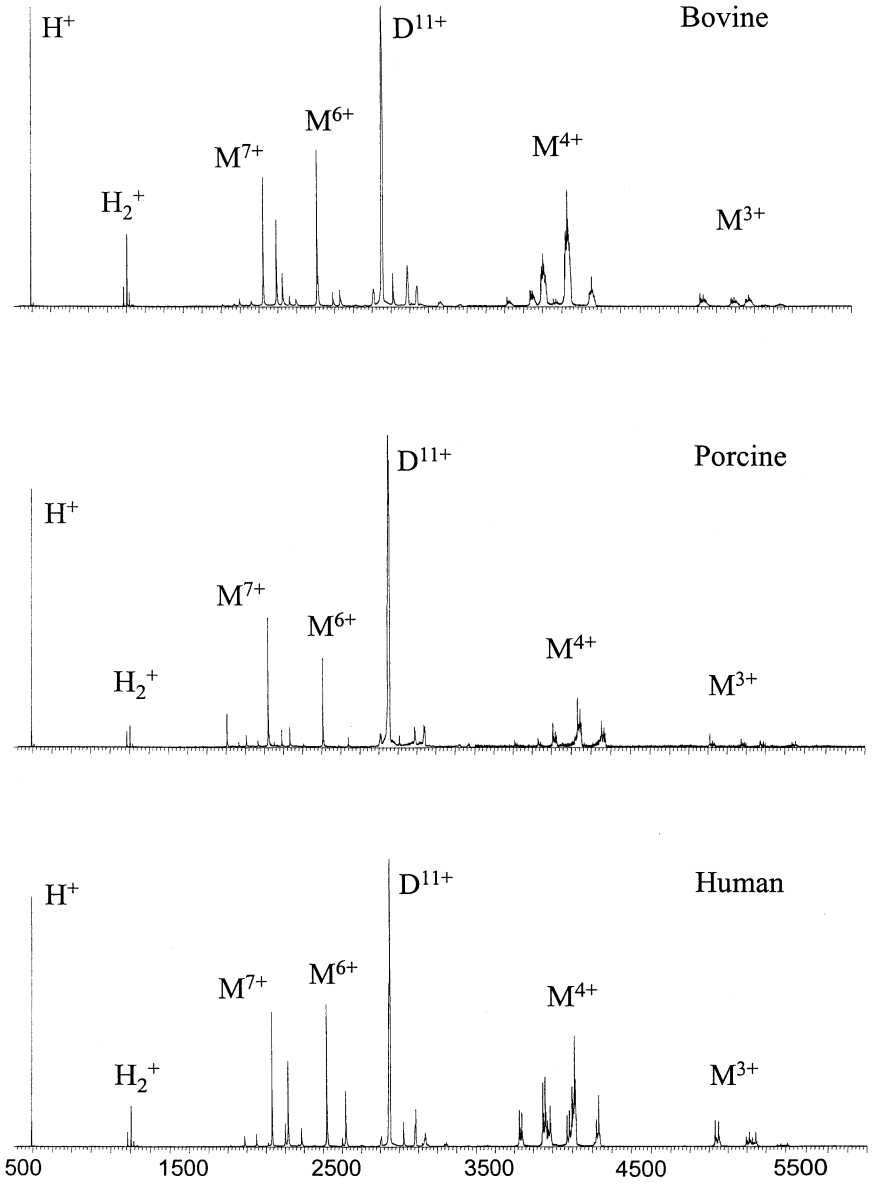


Fig. 1. CAD MS/MS spectra of the $[(\alpha H)(\beta H)]^{11+}$ ions of the intact holo-dimer of, from top to bottom, bovine, porcine, and human hemoglobin. M = monomer, D = dimer, and H = heme. The region $3500 < m/z < 6000$ was multiplied by 5.

fair share of the charge. The eleven-charged hetero holo-dimers dissociate preferentially into a seven-charged monomer concomitant with a four- or three-charged monomer. This disparate charge distribution over the two fragments, which are not that different in mass and other physical properties, is somewhat surprising. However, these observations are in agreement with earlier reported data on the gas-phase dissociation of homo-dimeric protein assemblies [21]. As described previously the disparate charge distribution may be rationalized by adapting a modified charged droplet fission model, first proposed by Smith

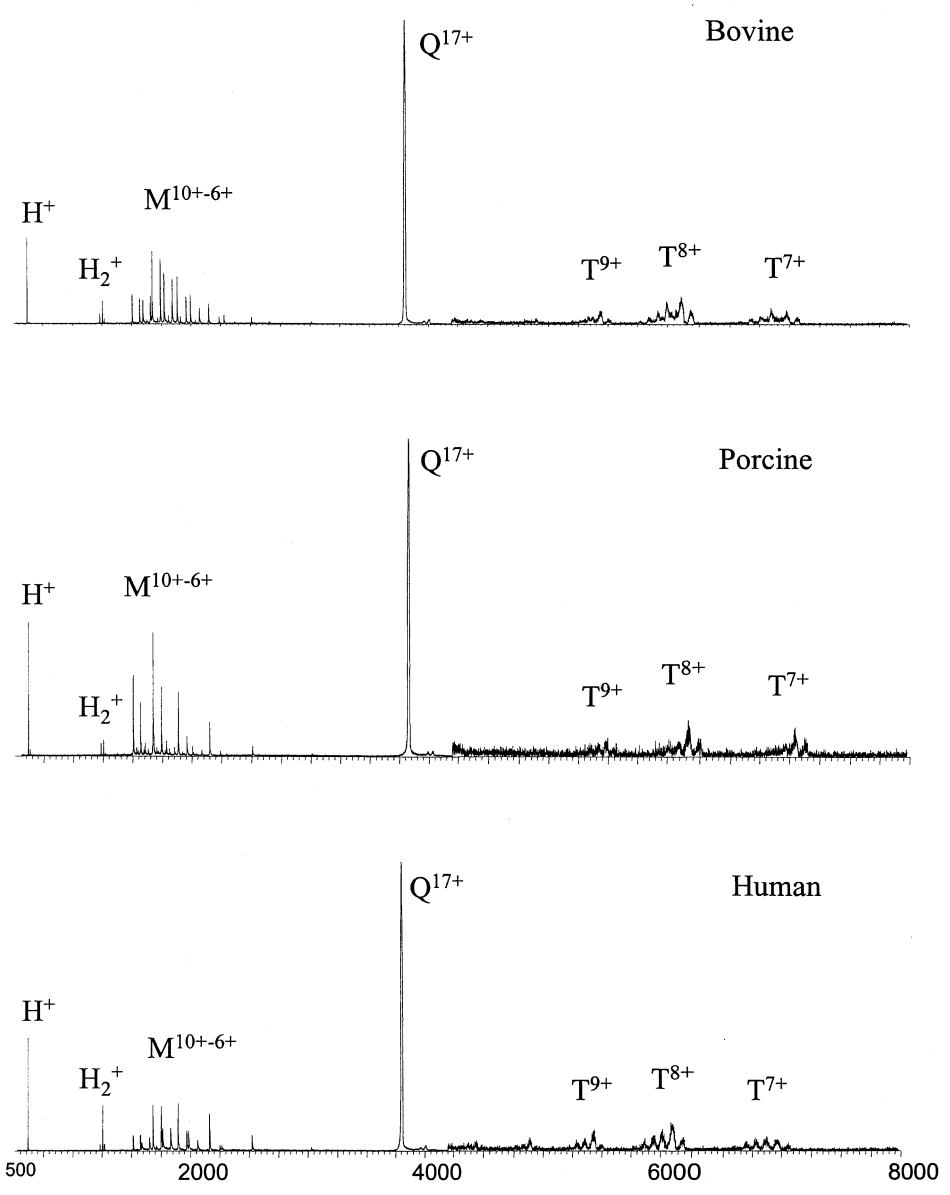


Fig. 2. CAD MS/MS spectra of the $[(\alpha H)_2(\beta H)_2]^{17+}$ ions of the intact holo-tetramer of, from top to bottom, bovine, porcine, and human hemoglobin. H = heme, M = monomer, T = trimer, and Q = tetramer. The region $4200 < m/z < 8000$ was multiplied by 10.

and co-workers [3,14]. For a more in-depth discussion we refer to the relevant literature [3,14,21].

To analyze the CAD MS/MS data in more detail the spectra were selectively transformed (i.e. over a selected *m/z* range) to "neutral" mass data. In Fig. 3 are shown the transformed data from the region $1500 < m/z < 2800$, i.e. the relatively high charged protein monomer region. In Fig. 4 are shown the transformed data from the region $3500 < m/z < 6000$, i.e. the low charged protein monomer region. These data allow a direct more detailed comparison between the spectra obtained for bovine, porcine, and human hemoglobin

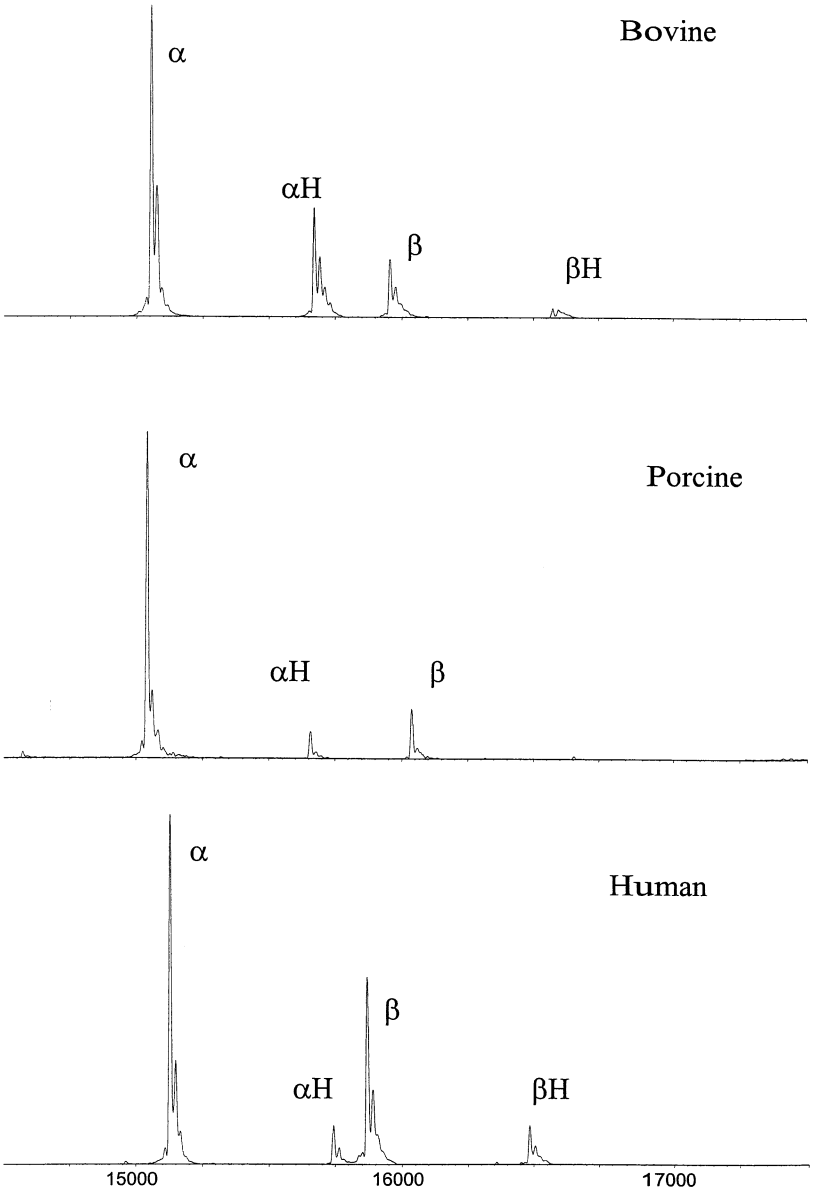


Fig. 3. Transformed data for the region $1500 < m/z < 2800$ (i.e. the relatively high charged protein monomer region of the CAD MS/MS spectrum of the $[(\alpha H)(\beta H)]^{11+}$ ions of the intact holo-dimer shown in Fig. 1. From top to bottom the transformed data are shown for bovine, porcine, and human hemoglobin, respectively. Besides the purely protonated species also some $M + Na^+$ adducts are observed.

dimers. Fig. 3 reveals that the prevalent high charged monomer fragments are the apo-α-chain and the holo-α-chain. For bovine and porcine hemoglobin this prevalence for high charge α fragment ions is strongest. The ratio of α to β fragment ions is 5 to 1 in bovine and porcine, whereas this ratio is 2 to 1 for human hemoglobin. Fig. 4 actually mirrors (and thus verifies) these observations; the prevalent low charged monomer fragments are the apo- and holo-β-chains, but maybe surprisingly also the β fragment with two hemes attached to it. This prevalence for the β-chain fragment ions is again most strikingly seen for bovine

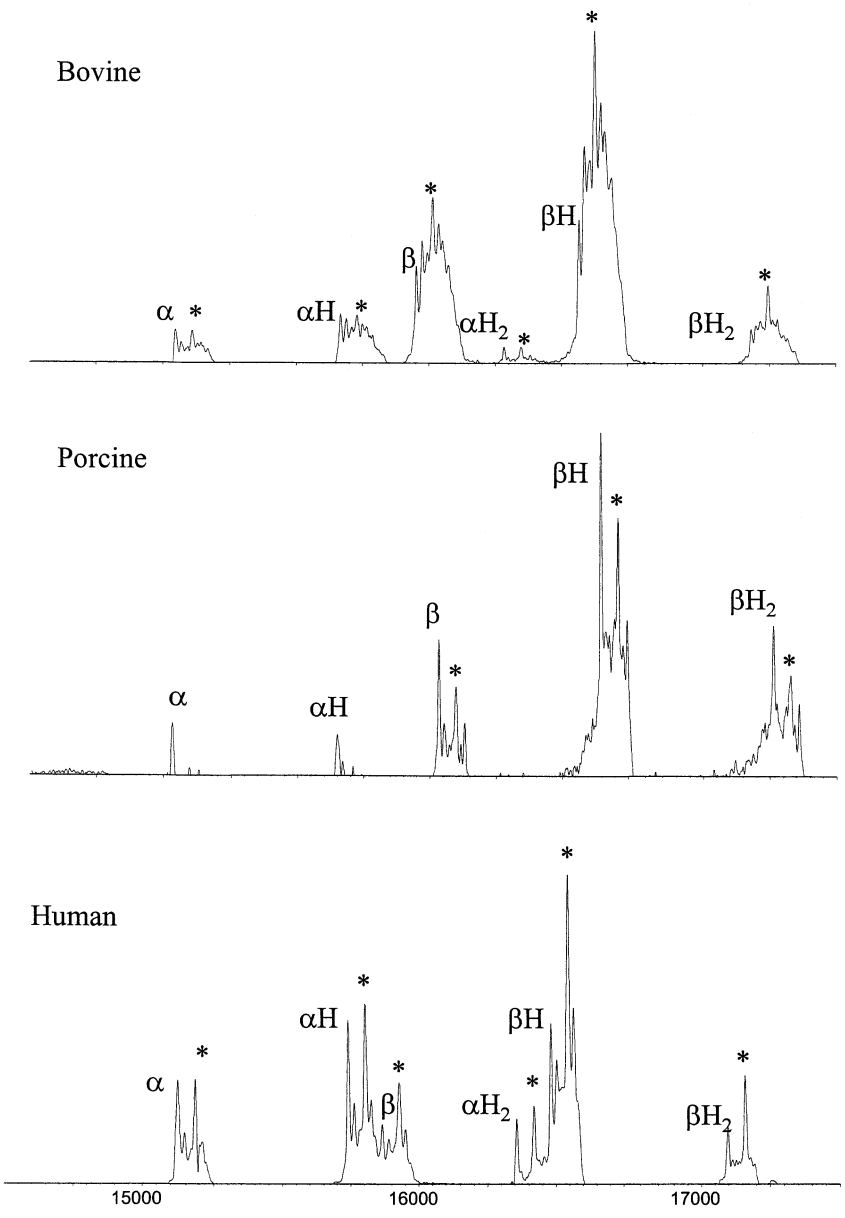
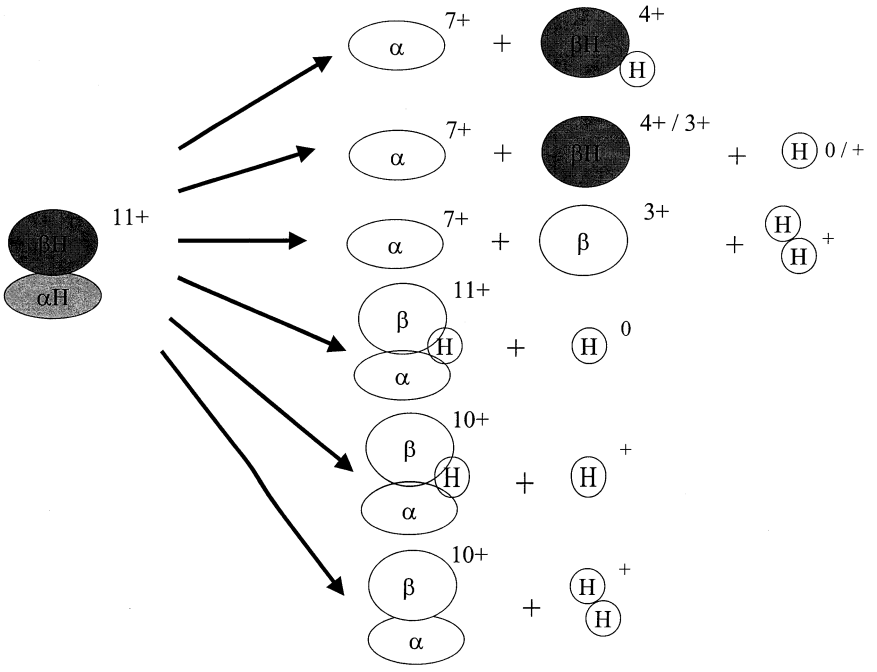


Fig. 4. Transformed data for the region $3500 < m/z < 6000$ (i.e. the relatively low charged protein monomer region of the CAD MS/MS spectrum of the $[(\alpha H)(\beta H)]^{11+}$ ions of the intact holo-dimer shown in Fig. 1. From top to bottom the transformed data are shown for bovine, porcine, and human hemoglobin, respectively. The peaks assigned with an asterisk are protein adduct ions originating from the addition of 60 Da (acetic acid) to the adjacent lower mass peak.

and porcine hemoglobin, somewhat less for human hemoglobin. The above-mentioned ratios between the α and β ions is now 1 to 5 for bovine and porcine and 1 to 2 for human hemoglobin. That the α-chain is prevalent in the high charge fragment ion region may be related to the solution-phase isoelectric points of the two chains. For all three hemoglobins the pI of the apo-α-chain is between 8 and 9, whereas the pI of the



Scheme 1. Possible reaction pathways for the most significant fragment ions originating from the $[(\alpha H)(\beta H)]^{11+}$ precursor ions in the CAD MS/MS experiments.

apo-β-chains is between 6.5 and 7, which would suggest that the α-chains more readily accommodate positive charges. However, if this explanation would be completely valid it would not predict the differences seen between the data of the hemoglobins originating from the three different species, as the pI difference between the apo-α- and apo-β-chains is very large in all three systems. It is expected that differences in the gas-phase structures of the three different assemblies are the cause for the differences in fragmentation patterns. Almost, all possible protein fragment ions are observed, also the monomer α- and β-chains to which two heme groups are attached. The observation of the βH_2 ions provides direct evidence that one route leading to apo-α-chain ions is direct expulsion from the intact hemoglobin holo-dimers precursor ions leaving the two hemes on the concomitant β fragment. In Scheme 1 is given a summary of the most important observed fragment ions together with the proposed pathways, in particular for the gas-phase disassembly of $[(\alpha H)(\beta H)]^{11+}$ ions.

Besides the "proven" direct expulsion of the $[\alpha]^{7+}$ ions, leaving the two hemes on the concomitant β fragment, the $[\alpha]^{7+}$ ions may also be formed indirect or by way of three-body dissociations, in which the third body is a neutral or singly charged heme or the singly charged heme-dimer (see Scheme 1). That the heme group is ejected from the complex either as a neutral or as singly charged fragment is also in agreement with the observation that holo-myoglobin $[MyoH]^{10+}$ ions may dissociate into $[Myo]^{10+}$ ions with the loss of a neutral heme or by way of the formation of $[Myo]^{9+}$ ions concomitant with a singly charged heme [24]. The observation that in the high *m/z* region (see Fig. 4) some of the most abundant species are the $[\beta H]^{4+}$ and $[\beta H]^{3+}$ fragment ions provides some indication that the three-body dissociation should be included in Scheme 1. The assumed three bodies are the $[\alpha]^{7+}$ ions, the $[\beta H]^{4+}$ or $[\beta H]^{3+}$ ions, and a neutral or singly charged heme. Unfortunately, using the present approach it is impossible to distinguish whether the three-body dissociations are

direct or occurring in sequential steps. Finally, the disassembly of the intact hemoglobin complex may also leave the hetero-dimer intact by the elimination of a neutral or singly charged heme or even a singly charged heme-dimer, as shown as the last three entries in Scheme 1.

4.2. CAD MS/MS hemoglobin holo-tetramers

First of all, the data shown in Fig. 2 reveal that CAD MS/MS may be performed on a Q-TOF on mass-selected ions exhibiting molecular weights above 60 kDa. In the CAD MS/MS data on the hemoglobin tetrameric protein assemblies primarily monomer species with between ten and seven charges and trimeric species with between ten and seven charges were observed. The nonobservation of dimeric species is already indicating that the gas-phase dissociation does not follow the most prevalent dissociation channel of the holo-tetramer in solution. Although, this behavior is a priori somewhat unexpected, the preference in the gas phase for the formation of trimeric species concomitant with monomeric species when compared to the formation of two dimeric species has been reported [3,23], albeit in experiments whereby dissociation was achieved without precursor ion selection (so-called nozzle-skimmer collision-induced dissociation). It is again somewhat surprising that the monomer fragment ions contain so many charges. The relatively small $[\alpha]^{10+}$ subunit strips away more charges that the concomitant $[\alpha\beta_2H_4]^{7+}$ trimer. Similar behavior has been observed before in the dissociation of tetrameric protein assemblies [3,14,23], rationalized again by a droplet fission model.

Following a similar approach as described for the dimer we transformed the $1500 < m/z < 3000$ region of the spectrum (see Fig. 5) separately from the region $4500 < m/z < 7500$ (see Fig. 6). As observed for the dimer, in the tetramer dissociation there seems to be a preference for the formation of highly charge α-chain over β-chain monomer ions. Comparing the transformed data of the high charge monomers of the holo-dimers (see Fig. 3) with the high charge monomers of the holotetramers (see Fig. 5) it is obvious that the intensity of the holomonomer ions from the tetramer is 3 to 4 times higher than from the dimer and there is even αH_2 and βH_2 present. Another observation is that for porcine the total ratio between α to β species is again 5 to 1. For human the ratio α to β is slightly from 2 to 1 for the dimer to 3 to 1 for the tetramer. But for bovine this ratio is changed from 5 to 1 for the dimer to 3 to 2 for the tetramer indicating that the likelihood for the expulsion of highly charged β-chains is much higher for the tetramer compared to the dimer. The transformed data of the relatively low charge trimer fragments mirror the signals observed in the high charge region, showing predominatly $\alpha\beta_2$ trimers. The observation of $\alpha\beta_2H_4$ trimer fragments indicates that an apo-α-chain may be directly expelled from the intact holo-tetramer. If we further focus on the formation of the $[\alpha]^{10+}$ ions originating from the $[(\alpha H)_2(\beta H)_2]^{17+}$ ions it is a priori only clear that the concomitant fragment(s) contain one α-chain, two β-chains, four hemes, and in total seven charges. As no dimeric species are detected in the fragment ion spectra it has to be assumed that the $[\alpha]^{10+}$ ions are formed concomitant with trimeric species, which are indeed observed in the fragment ion spectra. Scheme 2 gives an overview of the most abundant fragment ions and the possible routes by way of which they were formed. There is quite some similarity between Schemes 1 and 2, although no loss of neutral heme could be detected in case of the tetramer. In summary, a plethora of dissociation pathways is observed (see Scheme 2), all not very likely to occur in solution. These observations and those on the gas-phases disassembly of the holo-hetero-dimer ions lead to the conclusion that apparently the processes governing the dissociation pathways in the gas phase are quite different from those in solution.

5. Conclusions

The gas-phase disassembly of the holo-hetero-dimers and the holo-tetramers of bovine, porcine, and human hemoglobin have been investigated. The gas-phase disassembly of the hemoglobin originating from the three different species proceeds by way of

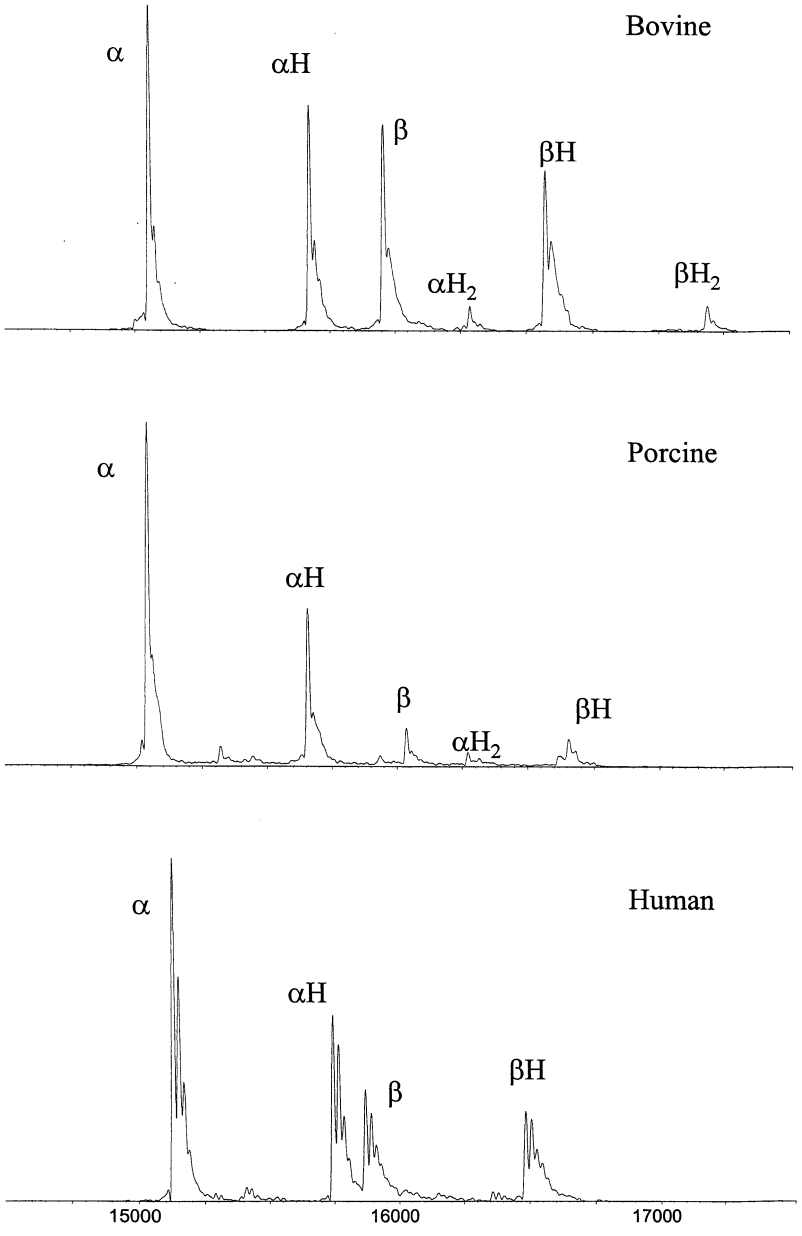


Fig. 5. Transformed data for the region $1500 < m/z < 3000$ (i.e. the relatively high charged protein monomer region of the CAD MS/MS spectrum of the $[(\alpha H)_2(\beta H)_2]^{17+}$ ions of the intact holo-tetramer shown in Fig. 2. From top to bottom the transformed data are shown for bovine, porcine, and human hemoglobin, respectively.

similar routes. Significant differences were observed in relative likelihood of the routes in between the species. For instance, the $\alpha H\beta H$ ions of hemoglobin from porcine dissociate primarily into highly charge α-chains, concomitant with much lesser charged apo-β, holo-β, or even β-H_2 ions, whereas the $\alpha H\beta H$ ions of human hemoglobin dissociate almost evenly into α- and β-chain ions. The $(\alpha H\beta H)_2$ holo-tetramer

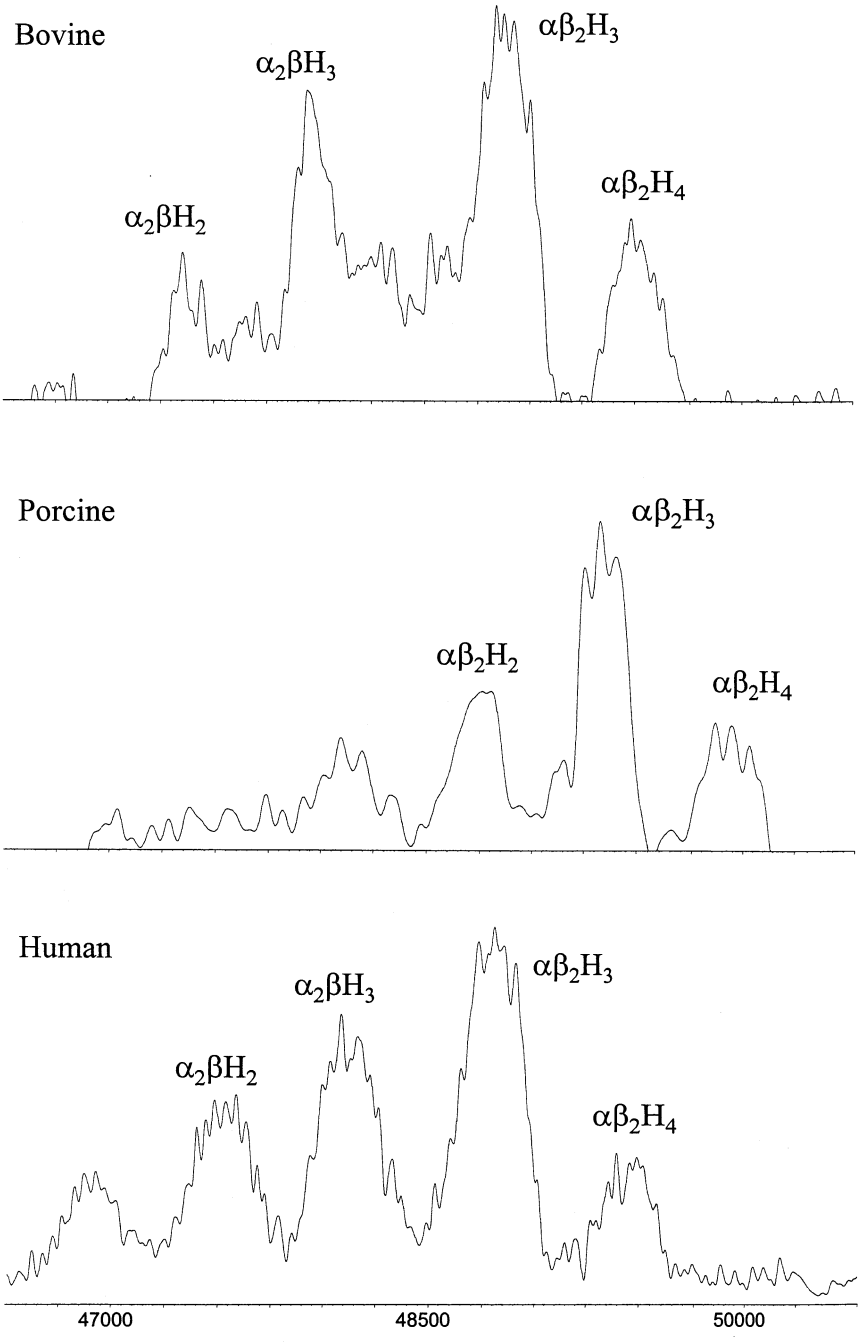
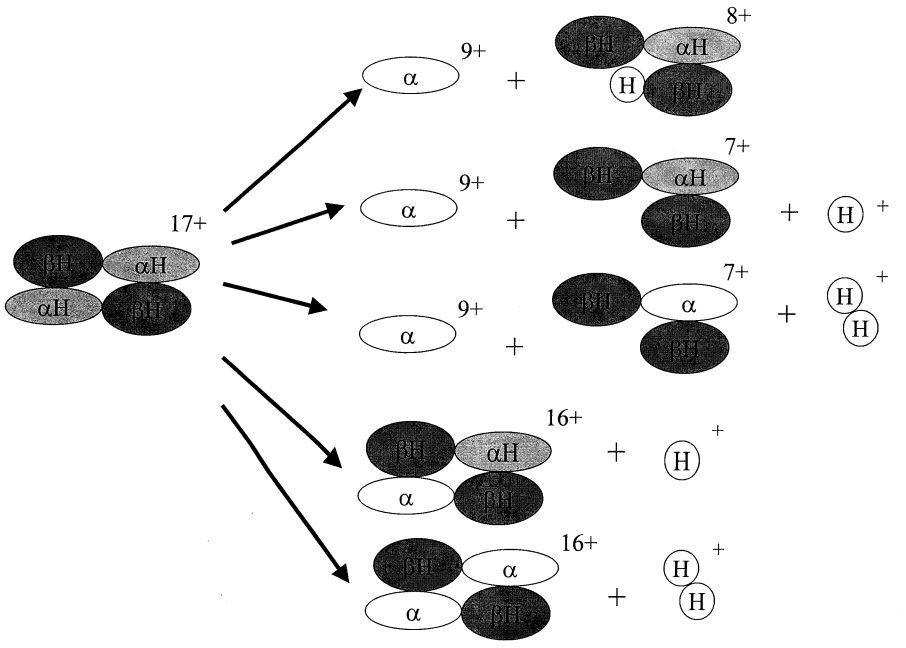


Fig. 6. Transformed data for the region $4500 < m/z < 7500$ (i.e. the relatively low charged protein monomer region of the CAD MS/MS spectrum of the $[(\alpha H)_2(\beta H)_2]^{17+}$ ions of the intact holo-tetramer shown in Fig. 2. From top to bottom the transformed data are shown for bovine, porcine, and human hemoglobin, respectively.



Scheme 2. Possible reaction pathways for the most significant fragment ions originating from the $[(\alpha H)_2(\beta H)_2]^{17+}$ precursor ions in the CAD MS/MS experiments. For simplicity the heme groups are in the fragment ions drawn attached preferentially to the β-protein, although in principle they could be bound anywhere to the ions.

ions dissociate primarily into highly charged α monomers and lesser charged $\alpha\beta_2$ tetramers, concomitant with the loss of none, one, or two heme groups (the latter also as a dimer). The disparate charge distribution following the dissociation is in agreement with previous observations on other protein assemblies, and may to some extent be explained by assuming a charge-droplet fission model. The most important conclusion from this study is that the observed pathways in the gas-phase disassembly of hemoglobin are in sharp contrast with well-characterized solution-phase routes. Therefore, the gas-phase structures and stability of the hemoglobin assemblies are likely quite different than the well-characterized solution-phase structures.

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