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Chemical cross-linking of the urease complex from *Helicobacter pylori* and analysis by Fourier transform ion cyclotron resonance mass spectrometry and molecular modeling

Elisabet Carlsohn[a,*], Jonas Ångström[a], Mark R. Emmett[b,c], Alan G. Marshall[b,c], Carol L. Nilsson[a]

[a] *Institute of Medical Biochemistry, Göteborg University, Box 440, SE-405 30 Göteborg, Sweden*
[b] *National High Magnetic Field Laboratory, Florida State University, 1800 East Paul Dirac Drive, Tallahassee, FL 32310-4005, USA*
[c] *Department of Chemistry and Biochemistry, Florida State University, Tallahassee, FL 32306, USA*

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Abstract

Chemical cross-linking of proteins is a well-established method for structural mapping of small protein complexes. When combined with mass spectrometry, cross-linking can reveal protein topology and identify contact sites between the peptide surfaces. When applied to surface-exposed proteins from pathogenic organisms, the method can reveal structural details that are useful in vaccine design. In order to investigate the possibilities of applying cross-linking on larger protein complexes, we selected the urease enzyme from *Helicobacter pylori* as a model. This membrane-associated protein complex consists of two subunits: α (26.5 kDa) and β (61.7 kDa). Three (αβ) heterodimers form a trimeric $(\alpha\beta)_3$ assembly which further associates into a unique dodecameric 1.1 MDa complex composed of four $(\alpha\beta)_3$ units. Cross-linked peptides from trypsin-digested urease complex were analyzed by Fourier transform ion cyclotron resonance mass spectrometry (FT–ICR MS) and molecular modeling. Two potential cross-linked peptides (present in the cross-linked sample but undetectable in α, β, and native complex) were assigned. Molecular modeling of urease αβ complex and trimeric urease units $(\alpha\beta)_3$ revealed a linkage site between the α-subunit and the β-subunit, and an internal cross-linkage in the β-subunit.

Keywords: Cross-linking; FT–ICR MS; FTMS; Urease; Molecular modeling

1. Introduction

The Gram-negative bacterium *Helicobacter pylori* is known to colonize the epithelium of the human stomach. In most infected patients (80%), *H. pylori* does not cause any clinical symptoms; however, the organism is associated with gastritis, gastric ulcers, and gastric cancer [1,2]. *H. pylori* urease (urea amidohydrolase, EC 3.5.1.5) is essential for colonization of the stomach because it allows the bacteria to survive under the acidic condition maintained by the gastric secretions [3]. Urease consists of two different subunits: α (26.5 kDa) and β (61.7 kDa) [4,5]. As in the case of other bacterial ureases, three (αβ) heterodimers form a trimeric assembly $(\alpha\beta)_3$. However, in *H. pylori*, this $(\alpha\beta)_3$ assembly further associates into a large (1.1 MDa) supramolecular complex composed of four $(\alpha\beta)_3$ units [6]. Expression of urease can account for as much as 15% of the total bacterial protein measured by dry weight [7]. Although urease is an intracellular enzyme found exclusively within the cytoplasm in bacteria and plants, about 30% of the *H. pylori* urease is associated with the outer membrane at the surface of the bacteria [8]. The enzyme may adhere to the surface upon spontaneous lysis of some bacteria followed by adsorption of the enzyme onto the outer membrane of the remaining intact bacteria [9]. Due to surface localization, urease has been implicated as a potential virulence factor and a putative vaccine candidate. However, there is still no effective vaccine against *H. pylori*.

In order to develop specific vaccine components, it is of great value to know the three-dimensional structure of the target protein, typically achieved by X-ray crystallography

* Corresponding author. Tel.: +46-31-7733049; fax: +46-31-416108.
E-mail address: elisabeth.carlsohn@medkem.gu.se (E. Carlsohn).

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and NMR spectroscopy. However, those techniques have limitations. The availability of high-quality analyte crystals is critical for X-ray crystallography and NMR is still limited to smaller protein complexes (60 kDa). Additionally, both techniques require large amounts (mg) of pure protein. Mass spectrometry is a sensitive technique with the ability to map protein domains from a small amount of sample (μg). Therefore, we report here the use of mass spectrometry to provide complementary structural information to aid in future vaccine design.

Mass spectrometry has been established as the standard method for identification of proteins and characterization of post-translational modifications. Fourier transform ion cyclotron resonance mass spectrometry (FT–ICR MS) [10] provides the highest mass resolution, highest mass resolving power, and highest mass accuracy (low ppm). When combined with electrospray ionization (ESI), FT–ICR MS [11,12] provides high analytical sensitivity requiring femtomoles or less of sample [13,14]. High resolution FT–ICR MS permits mass measurement of large peptides (>3000 Da) with a mass accuracy of a few ppm. Furthermore, FT–ICR MS offers two fragmentation techniques: electron capture dissociation (ECD) [15,16] and infrared multiphoton dissociation (IRMPD) [17,18], which provide complementary information about protein modifications [19].

A relatively new approach in mass spectrometry is higher-order structural determination of proteins and protein complexes. For example, hydrogen/deuterium exchange in combination with high resolution mass spectrometry has been used to map inter-subunit interactions in a HIV-associated protein [20], and several studies of intact non-covalent protein assemblies have verified the utility of mass spectrometry for determination of molecular composition in large protein complexes [21–24].

Chemical cross-linking monitored by mass spectrometric mapping is another method to determine spatial proximity between protein domains [25–30]. These techniques were recently reviewed by Beck et al. [31]. The general procedure includes cross-linking of functional groups (such as the ε-amino group of lysines) on the protein or proteins of interest with a homo- or bi-functional bridging agent, separation of cross-linked and non-cross-linked proteins (by chromatography or electrophoresis), and enzymatic hydrolysis of cross-linked protein. Finally, mass spectrometry is used to determine the point of cross-linking. When inter- and intra chain cross-links are formed in relatively large polypeptides, the number of potential cross-linked peptides increases. The high resolution and high mass accuracy of FT–ICR MS reduces the number of peptide combinations that can be matched to unique masses of the cross-linked sample. In an earlier study, FT–ICR MS analysis of cross-linked peptides from cytochrome *c* and lysozyme revealed low-resolution structural information about these proteins [32].

Here, we combine the unique capabilities of FT–ICR MS with chemical cross-linking and molecular modeling to study the macromolecular urease complex of *H. pylori*.

2. Experimental section

2.1. Strain and growth conditions

The *H. pylori* bacterial strain 17875 was obtained from the Culture Collection, University of Göteborg (CCUG), Sweden. The bacteria were stored at −80 °C in soy broth containing 15% glycerol by volume and grown on agar (14 g/l) containing 10% heat-inactivated fetal calf serum, under microaerophilic conditions for 2–3 days. The cells were scraped off, washed three times in phosphate-buffered saline (PBS) and stored at −80 °C until use.

2.2. Urease purification

The urease extraction procedure has been described previously [33]. In brief, thawed *H. pylori* cells (1 g, wet weight) were mixed with distilled water and centrifuged at 15,000 × *g* at 4 °C for 30 min. The supernatant was passed through a 0.2 μm Whatman filter and incubated with 20 mM NaH_2PO_4, 1 mM EDTA, and 1 mM mercaptoethanol. The extract was adjusted to pH 6.5 and urease was purified by two-step affinity chromatography by use of cellufine sulfate as the affinity medium. For column A, the equilibration and elution buffer was 20 mM NaH_2PO_4, 1 mM EDTA pH 6.5. Protein containing fractions were collected and screened for urease activity according to Rokita et al. [34]. Urease-positive fractions were pooled, adjusted to pH 5.5 and further purified over a second cellufine column, equilibrated with 20 mM NaH_2PO_4 and 1 mM EDTA, pH 5.5. Urease was eluted with 20 mM NaH_2PO_4, 1 mM EDTA, and 150 mM NaCl, pH 7.4. Purified enzyme was either digested in solution with trypsin, or cross-linked, separated by SDS-PAGE, and subsequently, digested in-gel with trypsin.

2.3. In-solution digestion of urease complex

For in-solution digestion, urease was incubated with sequence grade modified porcine trypsin (Promega, Madison, WI) with protein:protease ratio of 30:1 for 4 h at 37 °C. Dried samples were stored at −20 °C until use.

2.4. Cross-linking of urease α and β

The water-soluble homobifunctional *N*-hydroxysuccinimide ester, bis(sulfosuccinimidyl) suberate (BS^3) was purchased from Pierce Biotechnology (Rockford, IL). Cross-linking of the α- and β-subunits (approximately 200 pmol) was performed in 20 mM sodium phosphate (pH 7.5) containing 150 mM NaCl and a 200-fold excess BS^3 at room temperature for 30 min. The reaction was quenched with 1 M Tris–HCl (pH 7.8) (final concentration of 10 mM). The sample was concentrated by vacuum centrifugation. The cross-linked sample was separated by SDS-PAGE (10% Bis-Tris gel, Novex, San Diego, CA). Gels were stained with Coomassie blue (GELCODE® Blue stain reagent, Pierce

Biotechnology, Rockford, IL) and the protein bands were excised for further analysis.

2.5. In-gel digestion of cross-linked and monomeric urease

The method described by Shevchenko et al. [35] was applied with some minor modifications. Briefly, the gel pieces were destained by washing three times in 100 μl 25 mM NH_4HCO_3 in 50% CH_3CN for 30 min. Gel pieces were dried for 1 h in a vacuum centrifuge and incubated with 10–15 μl digestion buffer (50 mM NH_4HCO_3, 10 ng/μl trypsin) at 37 °C overnight. Peptides were extracted in 37.5% CH_3CN/1% formic acid (FA) (Sigma, St. Louis, MO) and the supernatant was evaporated to dryness in a vacuum centrifuge.

2.6. Accurate mass measurements of tryptic digests

The peptide digests were reconstituted in 15 μl 0.1% FA and enriched and desalted with C_{18} ZipTips (Millipore, Bedford, MA). The peptides were eluted in 10 μl of a 4:1 CH_3CN/H_2O solution containing 0.1% FA.

Mass measurements were performed with a homebuilt, passively shielded, 9.4 T FT–ICR mass spectrometer [36] equipped with dual switchable 50 μm i.d. fused-silica microelectrospray (μESI) emitters [37]. Dual emitters [38] allow for easy internal calibration of each spectrum [39]. The flow rate was 350 nl/min and the switching interval for the emitter containing calibrant solution (Agilent Technologies, Wilmington, DE) was 600 ms. Ions were accumulated externally in a storage octopole [40] followed by gated trapping in an open cylindrical ICR cell, chirp excitation [41,42] (72–480 kHz at 150 Hz/μs), and direct-mode broadband detection (512 K time-domain data). Hanning apodization and a one zero-fill were applied prior to fast Fourier transform followed by magnitude calculation. The experimental event sequence was controlled by a modular ICR data acquisition system (MIDAS) [43,44]. Internal calibration [45] was performed with calibrant ions of mass-to-charge (m/z) ratios 622.0289, 922.0097, and 1521.9714.

2.7. Data processing

Monoisotopic peak lists were generated by the THRASH algorithm [46] and subjected to database searches. The MASCOT search engine (Matrix Science, London, UK) was used to search the NCBInr database. Search criteria for all experiments included one missed cleavage for trypsin digest and oxidation of methionine as a variable modification. For peak lists generated from gel samples, modification of cysteine residues with acrylamide was added as a variable modification. Peptide mass tolerance was in all experiments set to ±50 ppm. Peak lists from of tryptic digests from the cross-linked and native samples were compared manually. Monoisotopic masses unique for the cross-linked sample were screened for cross-linker content.

2.8. Molecular modeling

For molecular modeling, the $(\alpha\beta)_3$ trimer of *H. pylori* urease was constructed from the coordinates of the (αβ) unit in PDB entry 1E9Z. The Quanta2000/CHARMm25 modeling package from Accelrys Inc. was used for the analysis.

3. Results

3.1. Urease purification

Native urease was separated from crude *H. pylori* cell extract by affinity chromatography. Urease screening of protein fractions and SDS-PAGE analysis followed by in-gel digestion of visualized protein bands and mass spectrometry analysis verified the presence of native highly purified urease protein (data not shown).

Cross-linking of active urease in solution with the lysine-specific cross-linker BS^3 revealed a new high molecular weight (HMW) band on the SDS-PAGE gel relative to the control (urease without BS^3) (Fig. 1). Peptide mass fingerprinting verified the presence of both urease α and β in the HMW band. Fig. 2 lists the amino acid sequence of both the urease subunits. The reactive lysines in the sequence are indicated by bold letters.

3.2. Identification of cross-linked peptides

After cross-linking and SDS-PAGE analysis, the complex was digested in-gel with trypsin and the desalted peptides were mass analyzed by FT–ICR MS (see Fig. 3). MS data

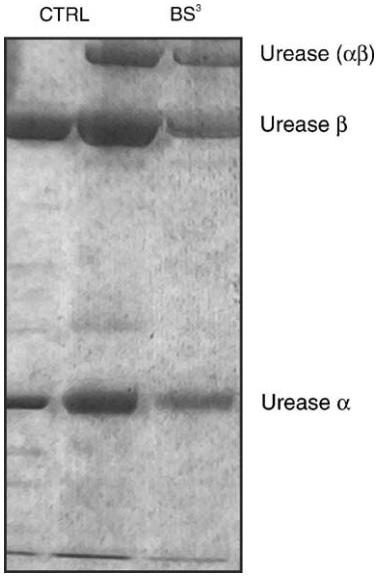


Fig. 1. SDS-PAGE of cross-linked urease. The control (CTRL) shows complex dissociation of native urease into the α- and β-subunits. Upon incubation with BS^3, a new high molecular weight (HMW) band appears, consisting of at least one α- and one β-subunit.

Urease α-subunit
MKLTPKELDKLMLHYAGELAKKRKEKGIKLNYVEAVALISAHIMEEARAGKKTAAELMQE
GRTLLKPDDVMDGVASMIHEVGIEAMFPDGTKLVTVHTPIEANGKLVPGELFLKNEDITI
NEGKKAVSVKVKNVGDRPVQIGSHFHFFEVNRCLDFDREKTFGKRLDIASGTAVRFEPGE
EKSVELIDIGGNRRIFGFNALVDRQADNESKKIALHRAKERGFHGAKSDDNYVKTIKE

Urease β-subunit
MKKISRKEYVSMYGPTTGDKVRLGDTDLIAEVEHDYTIYGEELKFGGGKTLREGMSQSNN
PSKEELDLIITNALIVDYTGIYKADIGIKDGKIAGIGKGGNKDMQDGVKNNLSVGPATEA
LAGEGLIVTAGGIDTHIHFISPQQIPTAFASGVTTMIGGGTGPADGTNATTITPGRRNLK
WMLRAAEEYSMNLGFLAKGNASNDASLADQIEAGAIGFKIHEDWGTTPSAINHALDVADK
YDVQVAIHTDTLNEAGCVKDTMAAIAGRTMHTFHTEGAGGGHAPDIIKVAGEHNILPAST
NPTIPFTVNTEAEHMDMLMVCHHLDKSIKEDVQFADSRIRPQTIAAEDTLHDMGIFSITS
SDSQAMGRVGEVITRTWQTADKNKKEFGRLKEEKGDNDNFRIKRYLSKYTINPAIAHGIS
EYVGSVEVGKVADLVLWSPAFFGVKPNMIIKGGFIALSQMGDANASIPTPQPVYYREMFA
HHGKAKYDANITFVSQAAYDKGIKEELGLERQVLPVKNCRNITKKDMQFNDTTAHIEVNP
ETYHVFVDGKEVTSKPANKVSLAQLFSIF

Fig. 2. Primary sequence of urease α- and β-subunit. The cross-linking agent BS^3 used in this study reacts with the amine groups of lysine and the N-terminus of the protein. The lysine residues are indicated with bold letters.

were compared to corresponding data from uncross-linked material (monomeric urease α and β and urease αβ complex). Tables 1 and 2 show the assigned peptides from each of these components. Peptide mapping of tryptic peptides from in-gel digested monomeric urease α and β yielded a primary amino acid sequence coverage of 76% for the α-subunit and 63% for β. Peptide assignments unique for native urease were for β-subunit K7-K20, G452-R476 and for α-subunit S228-K234, K52-R62, N115-K124, indicating that those segments may be involved in cross-linking. Of the

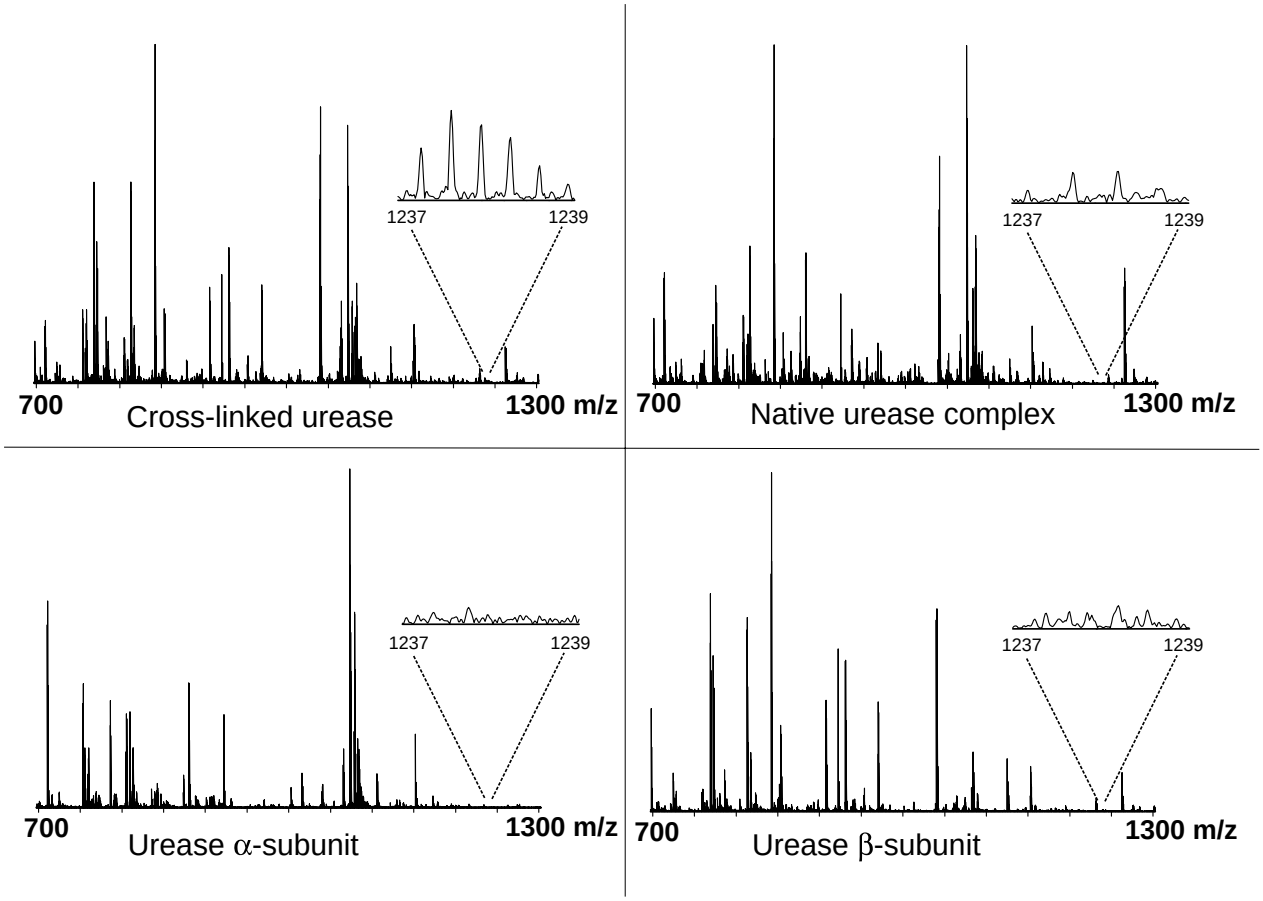


Fig. 3. FT–ICR MS analysis of cross-linked urease, native urease complex, monomeric α, and monomeric β. The inset shows the presence of *m/z* 1237.28^{3+} in the cross-linked sample. Of the 100 major peaks from the cross-linked urease, there was a complete overlap with the peaks from the mass spectrum of the native urease (α, β, or (αβ) complex).

Table 1
ESI–FT–ICR MS analysis of tryptic peptides from urease α-subunit

Observed *m/z*	Charge (*z*)	Peptide mass (Da)		Assignment	Error (ppm)
		Observed	Calculated		
609.383	1	608.376	608.376	I213-R217[a,b]	0.3
418.194	2	834.373	834.376	F176-K182[a,b]	3.7
840.371	1	839.364	839.367	S228-K234[a]	3.4
501.782	2	1001.550	1001.551	L166-R175[a,b]	1.1
508.311	2	1014.608	1014.611	L106-K114[a,b]	3.8
553.265	2	1104.515	1104.523	T53-R62[a,b]	7.7
561.266	2	1120.518	1120.518	T53-R62 (Met ox)[b]	0.5
566.776	2	1131.537	1131.541	N115-K124[a,b]	3.2
576.313	2	1150.612	1150.613	M95-R204[a,b]	1.4
579.834	2	1157.653	1157.652	R165-R175[a,b]	0.7
1249.622	1	1248.615	1248.613	K52-R62	1.2
630.824	2	1259.633	1259.636	N115-K125[a]	2.4
637.338	2	1272.661	1272.665	L11-R21[a,b]	3.4
664.866	2	1327.718	1327.721	S183-R194[a,b]	2.5
689.888	2	1377.761	1377.762	L93-K105[a,b]	0.2
879.963	2	1757.912	1757.913	E7-R21[a,b]	0.8
1065.055	2	2128.096	2128.099	L30-R48[a,b]	1.4
715.704	3	2144.090	2144.086	F176-R194[a,b]	1.6
753.086	3	2256.235	2256.237	L106-K125[a,b]	0.7
785.728	3	2354.163	2354.167	N133-R152[a,b]	1.8
809.774	3	2426.301	2426.299	G27-R48[a,b]	0.8
1083.524	3	3247.550	3247.545	T63-K92[a,b]	1.6
816.890	4	3263.529	3263.540	T63-K92 (Met ox)[a,b]	3.4

[a] Assigned peptides also detected in tryptic digest of native urease.
[b] Assigned peptides also detected in tryptic digest of cross-linked urease.

100 most abundant monoisotopic peptide masses found in the FT–ICR MS spectrum from cross-linked urease, there was a complete overlap with masses from analysis of peptides from monomeric α, β, and urease complex. Manual scanning of the unique monoisotopic masses present in the cross-linked sample but undetectable in α, β, and urease complex resulted in the identification of two potential cross-linked peptides, 3675.797 and 3708.817. These masses were examined for digested fragments containing intra- and intermolecular linkage. The cross-linking agent BS^3 produces either single residue modifications ($+C_8H_{12}O_3$) or inter-residue cross-links ($+C_8H_{10}O_2$). Moreover, because BS^3 modified lysine residues are not cleaved by trypsin. One criterion for the cross-linked peptides is that they contain at least one internal lysine. Two different peptide combinations with cross-linking between one α and one β unit were matched to 3675.797. To 3708.817, three different peptide combinations (either between two β-subunits or between one α and one β unit) were found (see Table 3). All combinations matched within 20 ppm. The large (for FT–ICR MS) mass deviation of the cross-linked peptides can be explained by their poor signal-to-noise ratio in the mass spectrum of the unseparated protein digest.

The method of choice in assigning one specific peptide combination to an unmatched one is MS/MS fragmentation analysis to reveal the primary sequence of the fragmented peptide. However, due to the low relative abundance of peptides of interest, no fragmentation analysis could be performed. We have previously attempted MS/MS characterization of cross-linked peptides. In a preliminary study of cross-linked urease by nano-LC-MS and MS/MS in a Qq-TOF (data not shown), the obtained sequence coverage was decreased compared to the coverage obtained by ESI–FT–ICR MS and we were unable to identify any cross-linked peptides.

In order to assign cross-linked peptides, we performed molecular modeling of the three-dimensional structure of urease. For 3708.817, internal cross-linking of K7-R22 to G395-K408 in the β-subunit between the lysines K20 and K403 was easily assigned from the structure of the (αβ) heterodimer. The distance between the linked lysines (K20 and K403) can range from 10 to 15 Å due to flexibility within the structure. Thus, it is possible for the BS^3 cross-linker (11.4 Å) to reach and react with the primary amines on the side chain of the lysines. To 3675.797, no peptide combinations were found within cross-linking distance in the (αβ) heterodimer. However, modeling of the trimeric urease unit $(\alpha\beta)_3$ identified a linkage site between K182 in the peptide F176-R194 within the α-subunit and K329 in the peptide fragment S327-R338 within the β-subunit (see Fig. 4).

4. Discussion

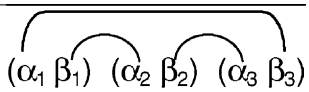
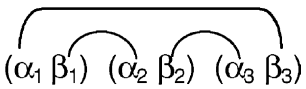
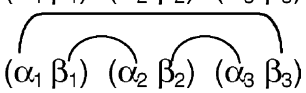
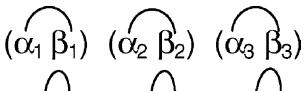
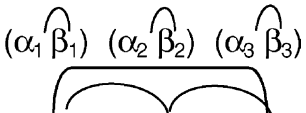
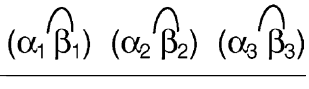
To apply structural information from cross-linking studies to the native protein, it is important that the cross-linking procedure occurs under native conditions. Identification of

Table 2
ESI–FT–ICR MS analysis of tryptic peptides from urease β-subunit

Observed (*m*/*z*)	Charge (*z*)	Peptide mass (Da)		Assignment	Error (ppm)
		Observed	Calculated		
773.450	1	772.443	772.4442	V369-R375[a,b]	2.2
1124.627	1	1123.620	1123.628	V560-F569[a,b]	6.8
1143.632	1	1142.625	1142.630	G502-R511[a,b]	3.8
697.846	2	1393.678	1393.684	S327-R338[a,b]	4.0
724.323	2	1446.631	1446.634	E8-K20[a,b]	2.1
732.319	2	1462.623	1462.629	E8-K20 (Met ox)[a,b]	3.7
772.376	2	1542.737	1542.739	A185-K198[a,b]	1.5
780.373	2	1558.731	1558.734	A185-K198 (Met ox)[a,b]	1.7
796.368	2	1590.722	1590.724	K7-K20 (Met ox)	0.7
853.408	2	1704.801	1704.799	Y487-K501[a,b]	0.8
635.651	3	1903.930	1903.932	A485-K501[a,b]	0.8
693.001	3	2075.980	2075.985	T269-K288[a,b]	2.4
698.332	3	2091.973	2091.980	T269-K288 (Met ox)[a,b]	3.0
768.733	3	2303.177	2303.180	Y409-K430[a,b]	1.1
787.772	3	2360.294	2360.297	V431-K451[a,b]	1.2
841.741	3	2522.200	2522.206	L23-K44[a,b]	2.4
1327.156	2	2652.297	2652.301	G452-R476[a]	1.1
890.438	3	2668.293	2668.293	G452-R476 (Met ox)	0.7
969.780	3	2906.319	2906.318	D526-K550[a,b]	0.5
975.114	3	2922.321	2922.313	D526-K550 (Met ox)[a,b]	2.6
1012.476	3	3034.406	3034.413	K525-K550[a,b]	2.3
763.608	4	3050.402	3050.408	K525-K550 (Met ox)[a,b]	2.0
1083.530	3	3247.568	3247.560	I339-R368[a,b]	2.6
816.893	4	3263.545	3263.556	I339-R368 (Met ox)[a,b]	3.0
1094.193	3	3279.557	3279.550	I339-R368 (2 Met ox)[a,b]	2.2
1157.914	3	3470.719	3470.713	E53-K83[b]	1.7
1629.549	4	6514.167	6514.272	N110-R176[a,b]	16.1

[a] Assigned peptides also detected in tryptic digest of native urease.
[b] Assigned peptides also detected in tryptic digest of cross-linked urease.

Table 3
Potential cross-linked peptides

Observed (*m*/*z*)	Charge (*z*)	Mass (*m*)		Error (ppm)	Assigned peptide	Lys–Lys cross-link	Distance (Å)[a]	Configuration[b]
		Observed	Calculated					
1226.2731	3	3675.797	3675.838	10.9	α F176-R194–β S327-R338	182–329	10.1	
			3675.866	18.5	α E159-K164–β Y487-R511	160–501	44.1	
						160–504	59.9	
1237.280	3	3708.817	3708.840	6.2	α T161-K182–β G99-K109	164–102	21.5	
			3708.856	10.3	β K7-R22–β G395-K408	20–403	14.9[c]	
			3708.876	15.8	β N521-K525–β L23-K49	524–544	37.7[d]	
			3708.876	15.8	β N521-K525–β L23-K49	524–544	43	

[a] Shortest possible cross-linking distance between internal lysines within the heterodimer (αβ) or trimer $(\alpha\beta)_3$ unit.
[b] Indicates if the shortest distance is within the monomeric unit (α), (β), the heterodimer (αβ), or within the trimeric $(\alpha\beta)_3$ unit.
[c] Due to the flexibility (no steric hindrance) of the internal lysines within these peptide chains, they can be as close as 10 Å.
[d] Shortest distance is through the trimeric unit.

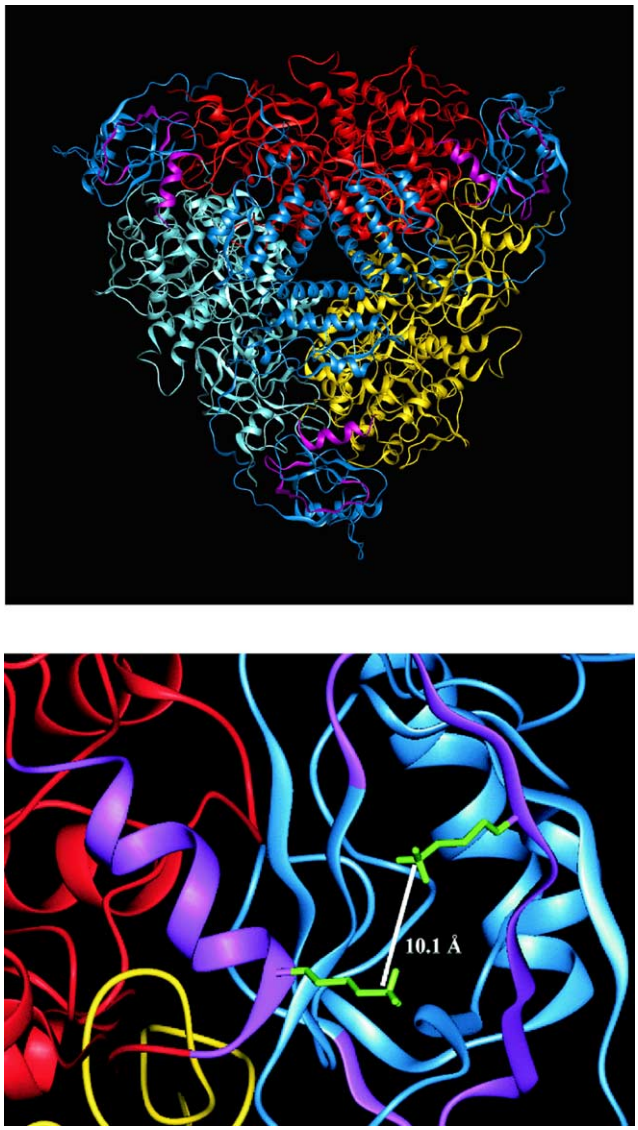


Fig. 4. View of the *H. pylori* urease $(\alpha\beta)_3$ trimer along the three-fold symmetry axis (top panel). The three α-subunits are colored blue while the β-subunits are shown in red, yellow, and light blue, respectively. Furthermore, the amino acid sequence 176–194 in the α-subunit and 327–338 in the β-subunit are shown in magenta in order to visualize one of the potential cross-linking peptide pairs (no. 1 in Table 3). In the lower panel, a close-up view of the upper right hand corner of the trimer is shown. Either of the lysyl residues αK182 and βK329 (colored green) may be modified by the cross-linker and subsequently cross-link with the other lysyl residue. The side chain N–N atom distance is 10.1 Å between these two residues.

a cross-link between αF176-R194 and βS327-R338 in the trimeric urease unit $(\alpha\beta)_3$ indicates that the complex is assembled into $(\alpha\beta)_3$ units. A previous study of *H. pylori* urease [21] demonstrated that the dodecameric urease complex $((\alpha\beta)_3)_4$ disassembles preferentially into $(\alpha\beta)_3$, both in solution and under gas-phase conditions. Furthermore, it was shown that re-association of denatured urease (monomeric α and β units) into $(\alpha\beta)_3$ also occurs in ammonium acetate at neutral pH. The identification of cross-linked peptides in a medium size protein complex such as the *H. pylori* urease is a difficult task. The under-representation of cross-linked peptides from urease in the ESI–FT–ICR mass spectrum may have several explanations. First and foremost, the larger the protein complex, the more potential binding sites for the cross-linking agent will be present. For cross-linking of the α- and β-subunit of urease, over 2000 different combinations, including intra-molecular cross-linking (within α- and β-subunits) and inter-molecular linkage between the units, are theoretically possible to achieve illustrating the potential heterogeneity of the mixture of cross-linked peptides. However, the number of groups within linking distance will limit the number of possible linkage combinations.

Cross-linked peptides, on an average, have larger masses than unmodified peptides and those larger peptides may have lower ionization efficiencies. Furthermore, the addition of a cross-linking agent to the peptide sequence may also influence the ionization efficiency of the peptide. Unambiguous identification of cross-linked peptides requires MS/MS analysis. One way to increase the recovery of cross-linked peptides would be to introduce a cross-linking agent with functional groups such as biotin which allows enrichment of labeled peptides. Kruppa et al. [47] have recently demonstrated a top–down approach in which cross-linked ubiquitin was analyzed by ESI–FT MS, and the cross-linked positions were localized by multiple fragmentation stages. That model is well-suited to small proteins but is difficult for proteins over 20 kDa. Compared to other cross-linking studies, no formation of single residue cross-links in which the cross-linking agent is attached to only one lysine residue on the protein surface was observed. It is noteworthy that both of the assigned cross-linked peptide pairs were surface-exposed.

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