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Cross-linking of Nitrogenase Components

STRUCTURE AND ACTIVITY OF THE COVALENT COMPLEX*

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The nitrogenase complex from *Azotobacter vinelandii* is composed of the MoFe protein (Av1), an $\alpha_2\beta_2$ tetramer, and the Fe protein (Av2), a γ_2 dimer. During turnover of the enzyme, electrons are transferred from Av2 to Av1 in parallel with the hydrolysis of MgATP. Using the cross-linking reagent, 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide, we have identified some of the properties of the complex between the two components. The cross-linking reaction was highly specific yielding a single apparent M_r = 97,000 protein. The amount of cross-linked product was essentially independent of whether MgATP or MgADP were in the reaction. Also, the amount was maximum at high ratios of Av2 to Av1.

The M_r = 97,000 protein was characterized by amino acid analysis and Edman degradation and was found to be consistent with a 1:1 complex of an Av2 γ subunit and an Av1 β subunit (the amino terminal serine subunit). The complex was no longer active in the nitrogenase reaction which supports, but does not prove, the requirement for dissociation of the complex after each electron transferred. Nitrogenase activity and cross-linking were inhibited in an identical way by NaCl, which suggests that electrostatic forces are critical to the formation of the electron transfer complex.

Biological dinitrogen reduction is catalyzed by the two protein complex, nitrogenase. The components of the complex are the MoFe protein, a $\alpha_2\beta_2$ tetramer of M_r = 225,000, and the Fe protein, a γ_2 dimer of M_r = 63,000 (3, 4). The MoFe protein contains the putative substrate reduction site which is a Fe_{5-7}:Mo:S_{6-9} cofactor. For substrate reduction, electrons are transferred from the Fe protein to the MoFe protein concomitant with the hydrolysis of ATP. An essential feature of the accepted model of nitrogenase turnover is the requirement for the Fe protein and the MoFe protein to dissociate after each electron transferred (5, 6). Because the substrates of nitrogenase require one or more *pairs* of electrons, the complex must undergo multiple cycles of association/dissociation for each mole of substrate reduced. A second prominent aspect of the reaction is the well documented structural change which occurs in the Fe protein upon binding MgATP and MgADP (3, 4).

A number of questions about the nature of the complex remain unanswered. For example, what is the symmetry of the complex, *i.e.* does the docking site on the MoFe protein involve both subunits? Can the complex reduce substrates without physically dissociating? Which amino acid residues are involved in the docking sites? What is the role of nucleotide binding in the docking process? Many of these questions can be evaluated by molecular model building once the crystallographic structures of the two components are known. On the other hand, model building will be greatly enhanced by having prior evidence for the docking sites. In addition some of these questions need to be considered for proteins under dynamic conditions. Towards these goals, we have used chemical cross-linking to study properties of the nitrogenase complex.

MATERIALS AND METHODS[1]

RESULTS AND DISCUSSION

A variety of compounds have been screened as potential cross-linking agents for the nitrogenase components; only the water-soluble carbodiimide, EDC,[2] which cross-links amino and carboxyl groups has proven promising. As is shown in Fig. 1, the EDC cross-linking reaction is highly specific for the nitrogenase complex. A *single* new protein band (apparent M_r = 97,000) was observed upon gel electrophoresis if both nitrogenase components were present in the reaction. In contrast, no cross-linking between the constituent subunits of the individual nitrogenase components was observed. When creatine phosphokinase (M_r = 45,000) was present in the reaction as part of an ATP regenerating system, it was not cross-linked either to itself or to the nitrogenase components. Furthermore, formation of the M_r = 97,000 product appears to require the active functional complex because only nonspecific, high molecular weight cross-linked material was observed with oxygen inactivated components (data not shown). The amount of cross-linking was independent of pH between 7 and 9 and of the EDC concentration from 1 to 50 mM. At higher concentrations of EDC (>100 mM), additional very

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[1] Portions of this paper (including "Materials and Methods," Figs. 1–6, and Table 1) are presented in miniprint at the end of this paper. Miniprint is easily read with the aid of a standard magnifying glass. Full size photocopies are included in the microfilm edition of the Journal that is available from Waverly Press.

[2] The abbreviations used are: EDC, 1-ethyl-3-(3-dimethylamino-propyl)-carbodiimide; Av1, component 1 or MoFe protein from *Azotobacter vinelandii*; Av2, component 2 or Fe protein from *A. vinelandii*; the Av1 α subunit is defined as the slower moving subunit on sodium dodecyl sulfate-gel electrophoresis and has threonine as its amino-terminal residue (1, 2); the Av1 β subunit is the faster moving subunit and has serine as its amino-terminal residue (1, 2); HEPES, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; PTH, phenylthiohydantoin.

large molecular weight material could be detected at the top of the electrophoresis gel.

Another line of evidence that the cross-linking reaction reflects the formation of an catalytically significant complex is the time dependence of the reaction. The results shown in Fig. 1 also indicate that the cross-linking is a rapid reaction with a $t_{1/2}$ of 3 min for the protein concentrations and ratio used in the figure. The reaction had excess EDC and was not limited by this reagent. EDC added after the cessation of cross-linking (30 min) did not increase the yield of M_r = 97,000 material.

One aspect of the nitrogenase model is that the component ratio as well as the protein concentration affect the amount of active complex and, thereby, the enzyme turnover. If the cross-linking reaction reflects the formation of the active complex, then the component ratio also might be expected to influence the rate of cross-linking. To investigate this further, the component ratio in the cross-linking reaction was varied from a 10-fold excess of Av1 to a 10-fold excess of Av2. The results are shown in Fig. 2. Throughout this 100-fold range of component ratios, only a single cross-linked protein band (M_r = 97,000) was observed. As the ratio of Av2:Av1 was increased, the amount of M_r = 97,000 material increased until the β subunit of Av1 was substantially depleted. Likewise, when the ratio of Av1:Av2 was increased (Fig. 2*B*), the amount of cross-linked material was limited by Av2 subunits. However, in contrast to the nearly complete loss of the Av1 β subunit, the Av2 subunits were decreased only 50–60%. Although we cannot exclude the possibility that 50% of our Av2 is inactivated by side reactions such that it can no longer react, it seems plausible that only one of the two subunits in each Av2 dimer is being cross-linked, whereas most of the Av1 β subunit is incorporated in the M_r = 97,000 material. This is also consistent with a specific interaction leading to cross-linking.

Perhaps the most surprising result of our studies was that the cross-linking reaction is essentially independent of the presence of nucleotides (see Fig. 3). Not only was the molecular weight and composition of the cross-linked material unrelated to the presence of nucleotides, but the amount of cross-linked material was similar or even somewhat less in the presence of either MgATP or MgADP. What makes this result so striking is that numerous other probes of the Fe protein structure uniformly show significant changes upon binding of nucleotides (3, 4). Under our experimental conditions of saturating nucleotide concentration, it is unlikely that the cross-linking reaction involves the nucleotide binding site or that nucleotide binding is prevented.

Because the EDC cross-linking reaction involves closely opposed carboxylic acids and amines, it is reasonable to assume that these groups may be part of ionic (salt) interactions which, in part, are responsible for the complex formation. Indeed, patches of acidic and basic residues have been postulated for "docking" sites in other electron transfer complexes and these residues can be cross-linked with EDC (*e.g.* Refs. 10–14). Studies by Watt and co-workers (15) and by Diets and Howard[3] have shown that nitrogenase activity is substantially inhibited by NaCl and other salts. Based upon modeling of the salt inhibition of enzyme activity, Diets and Howard[3] have concluded that salt prevents the formation of the Av1-Av2 complex required for enzyme turnover. Fig. 4 shows the results of salt effects on cross-linking of the nitrogenase complex. For comparison, the effect of salt on activity is also given. It is evident that both manifestations of the complex formation are equally inhibited and have a similar dependence on the salt concentration.

The effect of EDC and of the cross-linking reaction on the enzyme activity were investigated and the results are presented in Fig. 5. Just as the individual components were not cross-linked by incubation with EDC, they also were not inhibited. In contrast, the complex was rapidly inactivated by EDC. Furthermore, the inactivation occurred at a rate comparable to that of the cross-linking reaction, *cf.* results in Figs. 1 and 5. In accordance with the cross-linking reaction, the rate of inactivation was dependent upon the component ratio but independent of the presence of nucleotides. Thus, the cross-linked material does not appear to have enzyme activity. Although we cannot exclude the possibility that cross-linking prevents ATP hydrolysis or electron transfer (14), our results support the previous hypothesis that the complex must dissociate after each electron transferred (5, 6).

Identification of the number and types of subunits that compose the cross-linked complex has not been as simple as might have been expected for the reasons outlined above. Amino acid analysis (see Table 1) of the isolated complex was compared with theoretical values calculated using various ratios for the known Av1 and Av2 subunit compositions. The best fit of the data was for a 1:1 complex of an Av1 β subunit and an Av2 γ subunit. Because the amino acid compositions for the subunits are so similar (1, 16), unambiquous assignment of the ratio could not be made with confidence by this method.

Amino-terminal sequence analysis by Edman degradation gave a clear identity of the subunits in the M_r = 97,000 material. The results are shown in Fig. 6. At each cycle two phenylthiohydantoin-derivatives were observed which corresponded to the Av2 γ and Av1 β subunits. Equally importantly, no residues for the Av1 α-subunit were found. The calculated initial yields (based on M_r = 89,000) were 88 pmol (15%) for the β subunit and 712 pmol (29%) for the Av2 γ subunit, with repetitive yields of 93.6 and 91.2%, respectively. The ratio of the initial yields would suggest a cross-linked complex composed of 0.5 β-subunit and 1.0 γ-subunit. However, the low percent initial yield for both amino termini, which is often observed for material isolated using denaturing buffers or material from cross-linking reactions, leaves in doubt how reliable the ratio is when determined by Edman degradation.

Another approach to characterizing the composition of the complex is the analysis of the cross-linking reaction itself. Although molecular weights determined by denaturing gel electrophoresis may give anomalous results for branched (cross-linked) proteins, the molecular weight of 97,000 seems to best fit a ratio of 1:1 (M_r = 89,000). Furthermore, at saturating ratios of Av2, most of the Av1 β subunit was converted to the cross-linked complex whereas only ~50% of the Av2 subunits were cross-linked at saturating Av1. Since the Av2 subunits do not dissociate or exchange between native molecules,[4] the 50% cross-linking of the Av2 subunits is consistent with a 1:1 complex.

Thus, taking all of the results together, we favor the M_r = 97,000 cross-linked protein as being a 1:1 complex of the Av2 γ and Av1 β subunits. It should be emphasized that there is no evidence for the presence of the Av1 α subunit in the isolated cross-linked material. Identification of the specific amino acid residues involved in the cross-linking may help to determine the ratio. This is presently under way.

Our results provide several important conclusions about the interaction between the components of the nitrogenase complex, which may be related to the turnover of the enzyme during catalysis. First, at least part of the docking site for Fe

[3] T. Diets and J. B. Howard, manuscript in preparation.

[4] D. Ikeda and J. B. Howard, unpublished observations.

protein on the MoFe protein is in the β subunit. Of course, we cannot exclude a docking site between the two MoFe protein subunits where only the β subunit has the chemical cross-linking site.

Second, the docking site has some element of asymmetry in that only one of the two identical subunits of the Fe protein is cross-linked. Furthermore, because there is nearly complete incorporation of the β subunit, we conclude that the 2-fold symmetry of the MoFe protein (17) provides two separate binding sites and that each binding site interacts with a Fe protein dimer.

Third, a primary component of the docking recognition and binding energy comes from ionic interactions, at least some of which are between amino and carboxyl functions. The lack of cross-linking between subunits of the individual components, *i.e.* Av1 and Av2, suggests that ionic interactions may be more important in component docking than in subunit stabilization.

Fourth, the cross-linked complex appears to be inactive which supports the earlier hypothesis that the components must dissociate after each electron transferred.

Fifth, the individual components were neither inactivated nor cross-linked, whereas the complex was both rapidly cross-linked and inactivated. This is evidence for a specific interaction in the docking process.

Last, the docking of the two components is independent of the conformational changes induced by nucleotides bound to the Fe protein. This is quite significant, because the general belief is that these conformational changes are large if not global. However, whatever the scope of the structural change, it does not alter the region involved in binding to the MoFe protein. Since the docking site and the Fe:S cluster in the Fe protein might be expected to be contiguous for efficient electron transfer, an alternate model for the effect of ATP binding can be suggested. Namely, nucleotide binding could cause reorientation of the subunits with respect to each other without large intrasubunit conformational changes. In addition, if the nucleotide-dependent conformational change is a gating mechanism for electron transfer, then the component docking and electron transfer steps must be uncoupled to some degree.

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Materials, Methods, Tables and Figures for
Cross-linking of Nitrogenase Components: Structure and Activity of the Nitrogenase Complex
by
Andreas H. Willing, Millie Marshall-Georgiadis, Douglas C. Rees, and James Bryant Howard

MATERIALS AND METHODS

The manipulation of all reagents and proteins were done under anaerobic conditions using a Schlenk manifold with Ar and vacuum lines. The Ar was purified by passing over a BASF copper catalyst to reduce the traces of oxygen. Some experiments were performed inside an anaerobic glove box equipped with a Schlenk manifold. Nitrogenase components from *A. vinelandii*, Av1 and Av2, were purified and characterized as described previously (7,8). The H^+ reduction assay was used to monitor activity (7). For Av2, only protein with activity > 2750 nmols H_2 mg^{-1} min^{-1} and MgATP dependent Fe chelation of >3.7 Fe/mole was used. Av1 had an activity of > 2000 nmols H_2 mg^{-1} min^{-1}. Both proteins appeared as >95% single components on denaturing gel electrophoresis. The second, lower apparent molecular weight protein band frequently seen in the Av2 electrophoresis, see Figs. 1-4 is an anomaly of the electrophoresis system and is dependent upon the sample buffer components. Both bands have been isolated and found to be identical by peptide mapping and amino terminal sequencing. In the final purification step, a pH 8.0, 25 mM HEPES/KOH buffer was used for the Sephacryl S-300 gel filtration chromatography.

The concentration and amino acid composition of both native and reduced carboxymethylated proteins were determined using a Beckman 6300 analyzer (1). Samples were hydrolyzed in 6 M HCl for 24-72 hrs. at 110° C. A discontinuous gel electrophoresis system was used with N,N-bis(2-hydroxyethyl)glycine buffer (9). Amino terminal sequences were determined by repetitive Edman degradation using an Applied Biosystems 470A gas phase instrument with on-line PTH-amino acid analysis.

Cross-linking reactions with EDC were performed under anaerobic conditions in 2 ml serum vials. The standard reaction mixture was 500 µL and contained 25 mM, pH 8.0 HEPES/KOH buffer, 12.5 mM sodium dithionite, and protein (1-25 µM, depending on the experiment). The reaction was initiated by the addition of a 250 mM stock solution of EDC to give a final concentration of 12.5 mM. During the reaction, samples were taken either for enzyme activity assays or for gel electrophoresis. In the latter case, the reaction was terminated by diluting the protein into the usual sample preparation buffer containing 200 mM Na acetate.

The cross-linked protein was purified by gel filtration. A large scale reaction mixture (10 mg protein in 5 ml) was rapidly gel filtered under anaerobic conditions on Sephadex G-25 to remove EDC. The proteins were then dialyzed against 10 mM citric acid to remove metals followed by dialysis against pH 8.0, 50 mM ammonium acetate. The lyophilized proteins were dissolved in 3 mL of pH 8.0 TRIS/HCl buffer containing 0.2% sodium dodecyl sulfate and the disulfides were reduced anaerobically with 10 mM dithiothreitol for 1 hr. at 50° C. The proteins were separated by chromatography on a 150 x 2.5 cm column of Sephacryl S-300 resin using the same buffer. The cross-linked complex, Av1 subunits and Av2 subunits were identified by gel electrophoresis and the pooled materials were exhaustively dialyzed against 10% acetic acid to remove the denaturant.

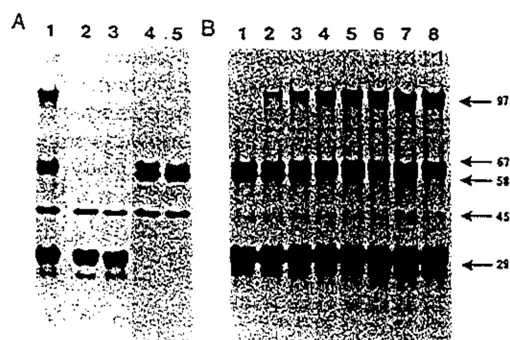

Figure 1. Specificity and time dependence of the cross-linking reaction between Nitrogenase components. Na dodecyl sulfate gel electrophoresis of samples from cross-linking reaction (see Methods). A. Specificity of the cross-linking reaction. The protein band at Mr=45,000 is creatine phosphokinase from the ATP regenerating system. Lane 1: Mixture of Av1 (1.6 µM) and Av2 (9.4 µM) incubated in presence of 12.5 mM EDC for 30 min. at 25° C. Lane 2: Av2 without EDC. Lane 3: Av2 incubated with EDC. Lane 4: Av1 without EDC. Lane 5: Av1 incubated with EDC. B. Time dependence of the cross-linking reaction. Lanes 1-8: Av1 (3.7 µM) and Av2 (22.1 µM) were incubated with 12.5 mM EDC. Samples were removed from the reaction mixture at 0, 0.5, 1, 2, 3, 5, 10, and 20 min., and were analyzed by gel electrophoresis.

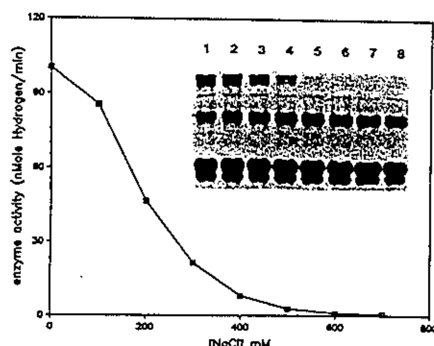

Figure 4. Salt dependence of nitrogenase activity and of the cross-linking reaction. Activity assays were done at a 10-fold molar excess of Av2 (47 µg Av1 and 166 µg Av2) in presence of the indicated concentrations of NaCl in 1 ml standard assays. In a separate experiment the nitrogenase components were incubated with 12.5 mM EDC for 5 mins. in presence of various concentrations of NaCl. Samples of the reaction mixtures were analyzed by Na dodecyl sulfate gel electrophoresis. The results are shown in the insert. Lane 1-8: 3.7 µM MoFe-protein and 22.1 µM Fe-protein were cross-linked for 12 min. under standard conditions in presence of 0 mM, 100 mM, 200 mM, 300 mM, 400 mM, 500 mM, 600 mM, and 700 mM NaCl, respectively.

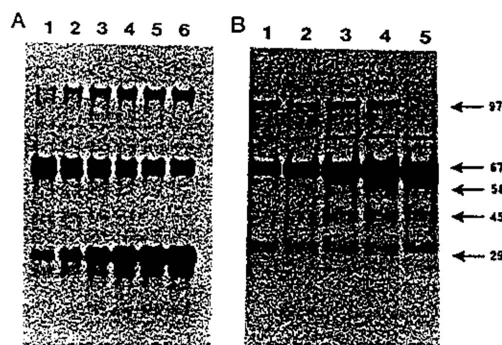

Figure 2. Component ratio dependence of the cross-linking reaction between Av1 and Av2. Na dodecyl sulfate gel electrophoresis of samples from cross-linking reaction. A. Reaction in the presence of an excess of Av2 under standard conditions. Lanes 1-6: 3.7 µM Av1 was reacted for 3 minutes with 3.7 µM, 7.4 µM, 11.2 µM, 22.1 µM, 29.8 µM, and 37.2 µM Av2, respectively. B. Reaction in the presence of an excess of Av1 under standard conditions. Lanes 1-4: 2 µM Av2 was reacted for 10 mins. with 2 µM, 4 µM, 8 µM, and 12 µM Av1. Lane 5: reaction mixture as in lane 4, but without EDC. Due to the large amount of MoFe-protein used in this experiment, minor impurities (<2%) become detectable, but as can be seen by comparison of lanes 4 and 5 the Mr=97,000 band is the only cross-linked product.

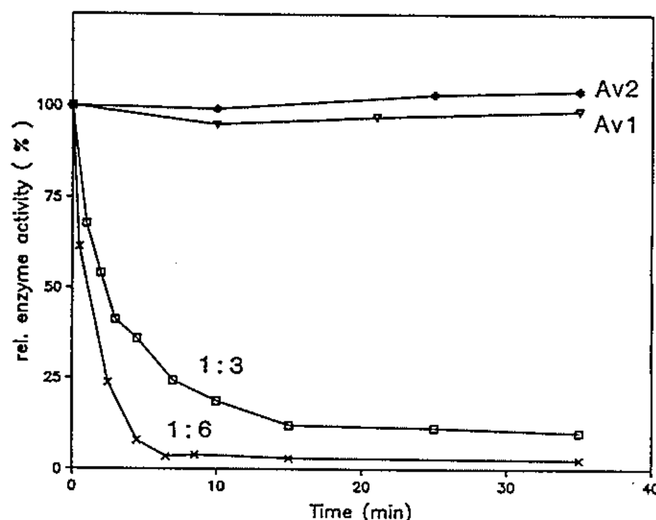

Figure 5. Component ratio dependence of nitrogenase activity under cross-linking conditions. 3.7 µM Av1 was incubated with 12.5 mM EDC and either a 3-fold molar excess (1:3) or a 6-fold molar excess (1:6) of Av2 under standard conditions. At the indicated time points samples were removed and assayed for nitrogenase activity in 1 ml standard H_2 evolution assays as described under methods. As a comparison the individual components (Av2, Av1) were incubated under identical conditions. In these cases samples of the reaction mixtures were assayed in presence of saturating concentrations of the other component.

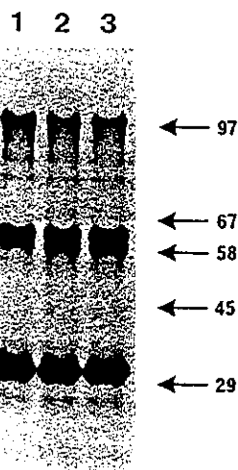

Figure 3. Cross-linking of nitrogenase components in presence and absence of nucleotides. 3.7 µM Av1 and 22.1 µM Av2 were incubated for 5 min. with EDC under standard conditions. Samples of the reaction mixture were analyzed by Na dodecyl sulfate gel electrophoresis. Lane 1: cross-linking without added nucleotide. Lane 2: cross-linking in presence of 5 mM MgATP. Lane 3: cross-linking in presence of 5 mM MgADP.

cycle	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	Ref.
α-chain	T	R	M	S	R	E	E	V	E	S	L	I	Q	E	V	L	E	V	Y	P	(1)
β-chain	S	Q	Q	V	D	K	I	K	A	S	Y	P	L	F	L	D	Q	D	Y	K	(1)
Av2 subunit	A	M	R	Q	C	A	I	Y	G	K	G	G	I	G	K	S	T	T	T	Q	(16)
found	S	Q	Q	V	D	K	I	K	A	S	Y	P	L	F	L	D	Q	D	Y	K	
	A	M	R	Q	C	A	I	Y	G	K	G	G	I	G	K	S	T	T	T	Q	.rm75

Figure 6. Amino terminal sequence of the crosslinked complex. Edman degradation for 20 cycles was performed as indicated in "Methods". For comparison, the amino terminal sequences for the isolated Av1 and Av2 subunits are given.

TABLE 1

Amino acid composition of the covalent complex

The purified complex was reduced carboxymethylated and duplicate samples were hydrolyzed for 24, 48, and 72 h as described in the Methods. Tryptophan is destroyed under these conditions and was not included for our analysis.

Calculated: From the published sequences of the nitrogenase subunits, the compositions of complexes for the indicated ratios of subunits and their exact molecular weights were calculated (1,2,16).

Found: The amino acid ratios which were obtained by amino acid analysis of the purified complex were normalized to the molecular weights of the three indicated complexes.

Difference: Percent difference between the found and the calculated compositions.

Amino acid	β,γ_1 - composition			β,γ_2 - composition			α,γ_1 - composition		
	Found	Calculated	Difference	Found	Calculated	Difference	Found	Calculated	Difference
Asx	86.3	87	- 0.8%	116.7	117	- 0.3%	82.4	75	+ 9.9%
Thr[a]	44.6	44	+ 1.4%	60.3	56	+ 7.7%	42.5	32	+32.8%
Ser[a]	34.3	35	- 2.0%	46.3	45	+ 2.9%	32.7	41	-20.2%
Glx	90.4	88	+ 2.7%	122.2	124	- 1.5%	86.2	86	+ 0.2%
Pro	34.4	34	+ 1.2%	46.6	42	+11.0%	32.8	28	+17.1%
Gly	69.3	67	+ 3.4%	93.7	95	- 1.4%	66.1	73	- 9.5%
Ala	59.6	57	+ 4.6%	80.6	85	- 5.2%	56.9	60	- 5.2%
Cys[b]	14.0	15	- 6.7%	19.0	22	-13.6%	13.4	16	-16.3%
Val	59.4	60	- 1.0%	80.4	86	- 6.5%	56.7	60	- 5.5%
Met	32.0	34	- 5.9%	43.3	48	- 9.8%	30.5	31	- 1.6%
Ile	43.9	45	- 2.4%	59.4	67	-11.3%	41.9	58	-27.8%
Leu	66.3	66	+ 0.5%	89.7	87	+ 3.1%	63.3	49	+29.2%
Tyr	25.7	26	- 1.2%	34.8	35	- 0.6%	24.5	30	-18.3%
Phe	34.3	36	- 4.7%	46.3	42	+10.2%	32.7	25	+30.8%
His	17.2	18	- 4.4%	23.3	20	+16.5%	16.4	13	+26.2%
Lys	56.2	56	+ 0.4%	75.9	73	+ 4.0%	53.6	55	- 2.5%
Arg	36.0	34	+ 5.9%	48.7	47	+ 3.6%	34.4	39	-11.8%
Difference, root mean squared			3.5%			8.0%			18.8%
Molecular weight		89,058			120,442			84,958	

a. Values are extrapolated to initial time of hydrolysis to account for destruction

b. Cysteine is determined as carboxymethylcysteine