Journal of Molecular Catalysis B: Enzymatic 61 (2009) 7–13

Contents lists available at ScienceDirect

Journal of Molecular Catalysis B: Enzymatic

journal homepage: www.elsevier.com/locate/molcatb

Solution NMR studies of acetohydroxy acid synthase I: Identification of the sites of inter-subunit interactions using multidimensional NMR methods

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ARTICLE INFO

Article history:
Available online 3 May 2009

Keywords:
Branched-chain amino acid biosynthesis
Nuclear magnetic resonance
Protein–protein interactions
Protein stabilizers
Proline

ABSTRACT

The novel multidomain organization in the multimeric *Escherichia coli* AHAS I (ilvBN) enzyme has been dissected to generate polypeptide fragments. These fragments when cloned, expressed and purified reassemble in the presence of cofactors to yield a catalytically competent enzyme. Structural characterization of AHAS has been impeded due to the fact that the holoenzyme is prone to dissociation leading to heterogeneity in samples. Our approach has enabled the structural characterization using high-resolution nuclear magnetic resonance methods. Near complete sequence specific NMR assignments for backbone H^N, ^{15}N, $^{13}C^{\alpha}$ and $^{13}C^{\beta}$ atoms of the FAD binding domain of ilvB have been obtained on samples isotopically enriched in ^2H, ^{13}C and ^{15}N. The secondary structure determined on the basis of observed $^{13}C^{\alpha}$ secondary chemical shifts and sequential NOEs indicates that the secondary structure of the FAD binding domain of *E. coli* AHAS large subunit (ilvB) is similar to the structure of this domain in the catalytic subunit of yeast AHAS. Protein–protein interactions involving the regulatory subunit (ilvN) and the domains of the catalytic subunit (ilvB) were studied using circular dichroic and isotope edited solution nuclear magnetic resonance spectroscopic methods. Observed changes in circular dichroic spectra indicate that the regulatory subunit (ilvN) interacts with ilvBα and ilvBβ domains of the catalytic subunit and not with the ilvBγ domain. NMR chemical shift mapping methods show that ilvN binds close to the FAD binding site in ilvBβ and proximal to the intrasubunit ilvBα/ilvBβ domain interface. The implication of this interaction on the role of the regulatory subunit on the activity of the holoenzyme is discussed. NMR studies of the regulatory domains show that these domains are structured in solution. Preliminary evidence for the interaction of ilvN with the metabolic end product of the pathway, viz., valine is also presented.

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1. Introduction

The biosynthesis of the branched-chain amino acids isoleucine, leucine and valine occurs in bacteria, plants and certain fungi [1]. Isoleucine and valine share a common set of enzymes in their biosynthesis. The biosynthesis of leucine involves recruitment of additional enzymes. The first step in the biosynthesis of these amino acids is catalyzed by acetohydroxy acid synthase (AHAS). The reaction catalyzed by AHAS is essentially an acyloin type condensation reaction, which involves a TPP dependent decarboxylation of a molecule of pyruvate, followed by an attack of the TPP-carboanion intermediate on a second molecule of pyruvate to yield acetolactate. Enterobacteria such as *E. coli* and *Salmonella* code for and express three isozyme forms of this enzyme, viz., AHAS I (*ilvBN*), AHAS II (*ilvGM*) and AHAS III (*ilvIH*) respectively. The activity and regulation of the isozyme forms of AHAS in *E. coli* has been the subject of detailed studies in the recent past. In terms of regulation, the AHAS I and AHAS III are feedback regulated by valine. It is known that regulation of branched-chain amino acid biosynthesis occurs through binding of valine to the regulatory subunit [2]. While structural information is available for the catalytic subunit of yeast AHAS [3] and regulatory subunit of AHAS III [4], no structural information is available for any of the catalytic subunits or for regulatory subunits of AHAS I and AHAS II. Our interest lies in understanding the structural basis for the regulation of the activity of these three isozymes of AHAS. Our efforts have been directed towards determining the structure of the regulatory subunits. Furthermore, we wish to determine the structural basis of the quaternary interactions between the regulatory subunits and catalytic subunits and the changes in these interactions upon valine binding that result in regulation of catalytic activity of this enzyme. We have executed a novel experimental approach to understand

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doi:10.1016/j.molcatb.2009.04.009

the determinants of activity of this enzyme. Our studies have relied on the application of high-resolution nuclear magnetic resonance (NMR) methods as well other spectroscopic methods such as circular dichroism to probe structure and structural changes associated with protein–ligand and protein–protein interactions. We have managed to clone, express and purify the TPP (α and γ domains) and the FAD binding (β domain) domains of ilvB, the catalytic subunit of AHAS I, as well as ilvN and ilvM, the regulatory subunits of AHAS I and AHAS II, respectively. An important point to bear in mind is that reconstitution of domains of ilvB with ilvN in the presence of cofactors yields a catalytically competent enzyme. We have also established sample conditions for the structural analysis of the regulatory subunits of AHAS I and AHAS II. The preliminary results of these studies are presented here.

2. Materials and methods

The cloning, expression and purification of ilvBα, ilvBβ, ilvBγ, ilvN have been described previously [5]. Here we describe the protocols used to purify ilvN and ilvM. It was also previously shown that the cloned ilvN had a N → Y mutation. In the studies here, this mutation has been reversed to generate the wild-type protein.

2.1. Expression and purification of ilvN and ilvM

The regulatory subunits of AHAS I and II, i.e., ilvN and ilvM, were cloned and expressed as a cytochrome b5 (cytb5) fusion protein [6]. The design and construction of the cytochrome b5 fusion protein has been described elsewhere. The DNA coding for ilvN and ilvM were ligated to the 3′-end of the cytb5 gene that was designed to contain a protease cleavage site at the C-terminal end of the cytb5 protein. Expression and purification of cytb5 fusion proteins has also been described previously [6].

2.2. Cleavage of ilvN and ilvM from cytb5 fusion protein and purification

2.2.1. Tev protease cleavage

Cleavage of ilvN from $cytb_5$–tev–ilvN was achieved by dialyzing the fusion protein into tev protease cleavage buffer comprising of 50 mM Tris, pH 8.0 with 0.5 mM EDTA and 10 mM DTT. The reaction was carried out at 22 °C for 16 h. The products of the cleavage reaction were analysed on a SDS-PAGE (15%) gel.

2.2.2. Purification of ilvN

ilvN was separated from cytochrome b5 and uncut cytb5–tev–ilvN fusion protein by preparative gel filtration using a S-100HR (Pharmacia) size exclusion chromatography column. The purity of the sample was checked on a SDS-PAGE (15%) gel as well as by mass spectrometry.

2.2.3. Factor Xa cleavage

Cleavage of ilvM from cytb5–fXa–ilvM was achieved by dialyzing the protein into the factor Xa cleavage buffer comprising of 50 mM Tris, pH 8.0, 100 mM NaCl and 1 mM $CaCl_2$. One unit of factor Xa was used to cleave each milligram of cytb5–fXa–ilvM. The reaction was carried out at 16 °C for 16 h. The products of the cleavage reaction were analyzed on a SDS-PAGE (15%) gel.

2.2.4. Solubilization and refolding of ilvM

A distinct turbidity was found to develop at the completion of the cleavage reaction of cytb5–fXa–ilvM. The precipitate was separated by centrifugation at 3000 rpm for 20 min at 4 °C. The pellet was washed thrice with 20 mM Tris buffer, pH 7.4. The protein could not be resolubilized in any of the standard buffers and was therefore unfolded in 6 M guanidine-hydrochloride and refolded by dialyzing

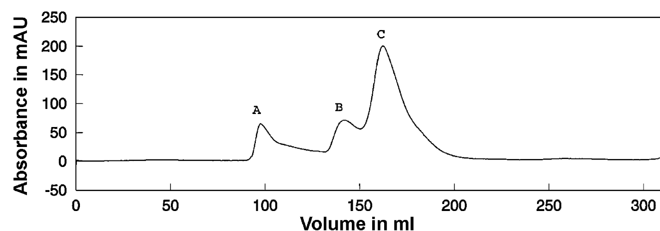

Fig. 1. Gel filtration chromatogram showing the elution profile of the products of the cytb5–tev–ilvn protein upon cleavage with tev protease. Fractions labeled A, B and C correspond to uncut fusion protein, ilvN and apocytochrome b5, respectively.

into 50 mM potassium phosphate buffer, pH 7.0, containing 100 mM NaCl, 5 mM DTT, 1 mM EDTA and 0.01% NaN_3. The purity of the sample was checked on a SDS-PAGE (15%) gel as well as by mass spectrometry.

2.2.5. Samples for NMR spectroscopy

Samples of ilvN for one-dimensional NMR spectroscopy were prepared by dialysing gel filtration purified samples of ilvN into a 20 mM phosphate buffer, pH 7.0 containing 20 mM NaCl, 1 mM EDTA and 0.01% sodium azide. Interaction of ilvN with valine was studied by addition of the latter to a final concentration of 5 mM to the above NMR sample. Samples of ilvM for one-dimensional NMR spectroscopy were prepared by dialyzing refolded samples of ilvM into 20 mM potassium phosphate buffer, pH 7.0, containing 20 mM NaCl, 5 mM DTT, 1 mM EDTA and 0.01% NaN_3 supplemented with 50 mM arginine, 50 mM glutamate and 20 mM proline.

2.3. Domain reconstitution

2.3.1. Enzyme assays

For enzyme activity studies the proteins were dialyzed into a 50 mM potassium phosphate buffer, pH 7.8, containing 100 mM KCl, 1 mM DTT, 1 mM EDTA, 40% glycerol and 0.01% NaN_3. The

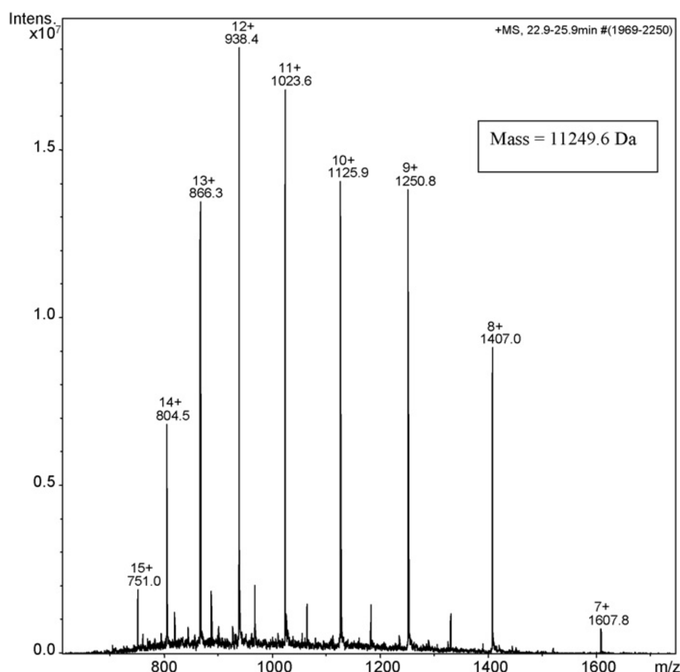

Fig. 2. LC-ESI mass spectrum of ilvN. The measured mass exactly corresponds to the calculated mass of the protein when produced as the cytochrome b5 fusion protein.

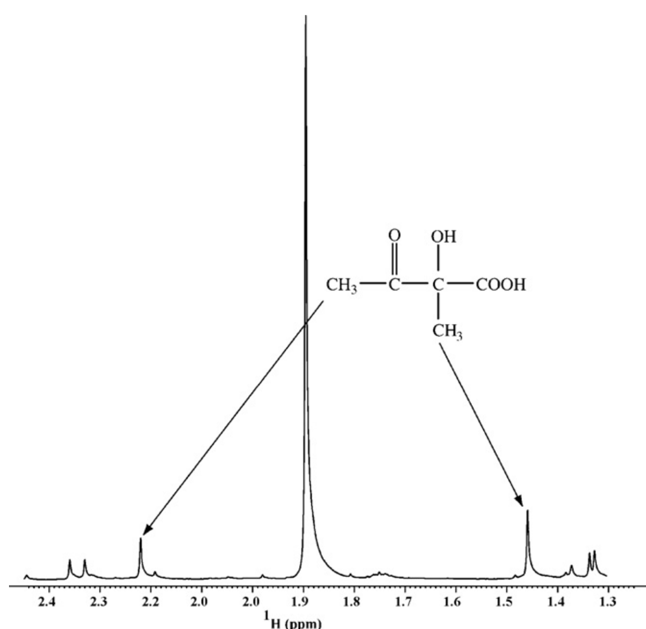

Fig. 3. One-dimensional NMR spectrum of the reaction mixture showing the presence of acetolactate. The intense peak at 1.9 ppm corresponds to acetate, which is produced as a side product in the AHAS reaction. The resonance signals originating from the methyl groups of acetolactate are indicated by arrows. The reaction was carried out at 50 μM concentration of each of the domains of ilvB and ilvN.

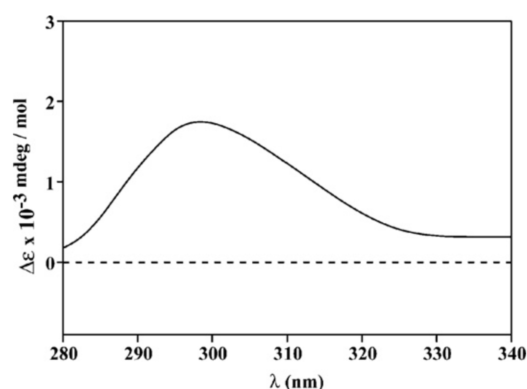

Fig. 4. CD spectrum of the product of the enzyme reaction catalyzed by the ilvBα–ilvBγ complex. The spectrum was acquired 8 h after the initiation of the enzyme reaction. The appearance of a positive CD signal with maxima at 300 nm is characteristic of S-acetolactate, thereby confirming that the absolute configuration of the reaction product is same as that obtained from the native AHAS I.

concentrations of the purified proteins were calculated from the absorbance values measured at 280 nm using molar extinction coefficients (ε) computed from primary sequence information of each protein. Products of enzyme reactions were monitored by both direct observation using NMR spectroscopy and circular dichroism or by colorimetric methods [2].

Fig. 5. CD spectra of the reconstituted domains of ilvB, the domain and subunits combinations being ilvBα–ilvBγ (A), ilvBα–ilvBγ–ilvN (B), ilvBα–ilvBβ–ilvBγ (C) and ilvBα–ilvBβ–ilvBγ–ilvN (D). Overlaid in each spectrum is the calculated spectrum of the complex in absence of any interaction between the individual components of the mixture.

2.3.2. CD spectroscopy

CD spectra of the enzyme reaction products were recorded using a JASCO-715 spectropolarimeter. The spectra were acquired in the wavelength range of 340–260 nm at a scan rate of 100 nm/min using a 2 mm path length cylindrical quartz cuvette.

CD spectra of ilvBβ, ilvBγ, ilvN and ilvM were acquired after the removal and separation of the proteins from the cytb5 fusion tag. The spectra were acquired in a wavelength range of 300–190 nm. The concentrations of all the proteins were in the range of 25–30 μM. All protein samples were made in 50 mM potassium phosphate buffer, pH 7.0, containing 100 mM KCl.

2.4. NMR studies of ilvBα, ilvBβ, ilvN and ilvM

All NMR data were acquired using samples in the concentration range between 0.15 and 0.2 mM. All NMR spectra were acquired on a Bruker 700 MHz instrument equipped using a cryogenically cooled probehead fitted with *Z*-axis gradient coil. All spectra were acquired at 303 K and referenced to internal water.

2.5. Mass spectrometry

ESI mass spectra were acquired on a Bruker Daltonics Esquire 3000 Plus Ion Trap Mass Spectrometer. Samples were infused into the mass spectrometer through a reverse-phase C-18 column. The data were acquired over a m/z range of 100–2000 in positive ion mode and analyzed using Esquire analysis software.

3. Results

3.1. Purification of ilvN and ilvM

The gel filtration chromatogram showing the separation of ilvN from cytochrome b5 is shown in Fig. 1. IlvN elutes from the column in a single fraction and exhibits a molecular mass that is larger than that of apocytochrome b5. Previous studies have shown that monomeric apocytochrome b5 behaves as a ~25 kDa molecule [6]. Thus it is reasonable to expect that ilvN elutes as a dimeric species from the gel filtration column. Fig. 2 shows the mass spectrum of the purified ilvN protein. From the measured mass it is clear that the earlier N → Y mutation has been reversed. The measured mass corresponds to the exact mass that is expected for the protein generated from the cytb5 fusion protein.

3.2. Reconstitution of domains

Reconstitution of ilvBα, ilvBβ, ilvBγ and ilvN results in a catalytically competent enzyme [5]. Fig. 3 shows the one-dimensional NMR spectrum of the products of the reaction at a fixed enzyme concentration (50 μM). The disappearance of pyruvate (δ 2.34 ppm) and the appearance of a strong signal for acetate (δ 1.9 ppm) and weaker signals for acetolactate (δ 2.23 ppm, 1.46 ppm) and acetoin (δ 1.33 ppm) indicates that the reconstituted domains form an enzymatically active complex. The absolute configuration of the acetolactate formed in the above reaction was studied by CD spectroscopy. The CD spectrum showed a positive Cotton effect (Fig. 4), indicating that the product formed is of the same absolute configuration as the acetolactate produced by native AHAS I. Circular dichroism spectroscopy also showed that the domains of ilvB and ilvN interact structurally. Fig. 5 shows the CD spectra of the protein complexes formed by (A) ilvBα–ilvBγ, (B) ilvBα–ilvBγ–ilvN, (C) ilvBα–ilvBγ–ilvBβ and (D) ilvBα–ilvBβ–ilvBγ–ilvN domains and subunits, respectively. Overlaid in each spectrum is the calculated spectrum that would result from individual contributions of each component of the complex in the absence of physical interaction. A qualitative change in the CD spectrum (distinct from the calculated spectrum) was observed upon addition of each protein. These changes suggested that the domains and the regulatory subunit interact upon reconstitution. The CD spectrum of the complex containing all the domains of ilvB as well as the regulatory subunit ilvN, is similar to the CD spectrum of the holoenzyme of *E. coli* AHAS II [7].

3.3. Interaction of ilvN with ilvBα and ilvBβ

The interaction between ilvN and the domains of ilvB was carried out using circular dichroism spectroscopy and NMR spectroscopy. Addition of ilvN to ilvBα, ilvBβ and ilvBγ showed that ilvN interacts to a significant extent with the α and β domains of ilvB. This inference is based on observed changes in the CD spectrum of each individual domain in the presence of ilvN. NMR spectroscopic studies have shown that the resonance positions of backbone HN nuclei of several residues change in the presence of ilvN. There is no apparent change in neither the CD spectra nor the NMR spectra in the case of ilvBγ in the presence of ilvN. This is taken as a clear indication that ilvBγ does not interact with the regulatory subunit [5].

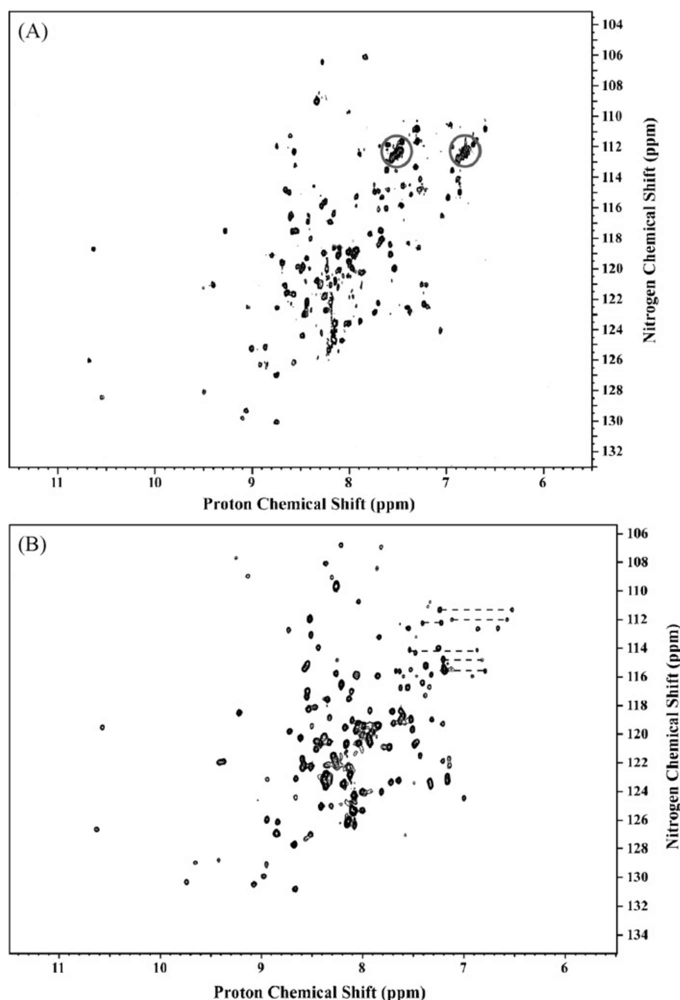

Fig. 6. Two-dimensional $^{1}H-^{15}N$ correlation spectra of ilvBβ obtained in the absence (A) and presence (B) of proline. The resolved signals emanating from the side-chain amide groups of asparagines and glutamines (connected by broken horizontal lines) is a clear indicator of the effectiveness of proline in preventing protein aggregation, particularly at the high concentrations used for NMR structural studies.

3.4. Backbone and side-chain resonance assignments of ilvBβ

The NMR studies of ilvBβ have been described in detail elsewhere [5]. As mentioned earlier, the subunits of AHAS are prone to dissociation resulting in a labile holoenzyme. Our studies have shown that the individual domains of ilvB are prone to aggregation at the concentrations used for NMR structural studies. We have been able to overcome this problem of aggregation by carefully monitoring solubility of the protein by addition of various solubility enhancers and stabilizers. Of the various conditions explored, inclusion of proline at a concentration of 20 mM provided samples of higher concentration that were not prone to aggregation [5]. Fig. 6 shows the ^{1}H–^{15}N correlation spectra for ilvBβ in the absence and presence of proline. Similar results were obtained in the case of ilvBα. In the case of ilvBβ we were able to obtain sequence specific assignments (H^N, ^{15}N, $^{13}C^{\alpha}$ and $^{13}C^{\beta}$) for 92% of the residues.

3.5. Interaction of FAD with ilvBβ

Addition of FAD to ilvBβ results in spectral changes consistent with FAD binding to this domain at a single site. On the basis of the assignments obtained above we have identified that the residues of ilvBβ that exhibit significant changes in chemical shifts upon addition of FAD are G40, T63, L64, L81, G82, R105, R109, A110, and D146. It is interesting to note that these are the identical residues in the catalytic subunit of yeast AHAS that are involved in FAD binding. The conservation of FAD binding residues in sequence and structure strongly suggest that the tertiary structure of this domain is highly conserved when compared to the yeast counterpart [5].

3.6. Interaction of ilvN with ilvBβ

Changes in the NMR spectrum of ilvBβ upon addition of ilvN allowed easy identification of residues on ilvBβ involved in binding ilvN. In all, six residues showed significant shifts. The residues of ilvBβ that interact with ilvB have been mapped onto the FAD binding domain of the yeast AHAS. It was clearly seen that ilvN acts at a site on ilvB that is close to the FAD binding site. Furthermore it is also seen to interact at the α/β inter-domain interface within a catalytic subunit. This is consistent with the observation of changes of lower magnitude in the spectra of ilvBα in the presence of ilvN [5].

3.7. Structural studies of ilvN and ilvM

So far only the regulatory subunit of AHAS III, i.e., ilvH [4] has been structurally characterized. Inspite of this very little is known of the structural basis for the all important regulatory interaction between the end products of the metabolic pathway and the regulatory subunits. Previous attempts to structurally characterize the regulatory subunits of AHAS I and AHAS II were met with failure due to the propensity of the purified proteins to aggregate in solution. Recently we have met with some success in producing proteins that are amenable to structural characterization. Fig. 7 shows the one-dimensional NMR spectrum of ilvM. The appearance of resonance lines above 9.0 ppm and around 0.0 ppm strongly indicate that the protein is well structured in solution. While the exact oligomeric state of this protein is not known, comparison of the spectrum with other protein of similar molecular weight, suggest that the protein is present as a monomer under the conditions of the NMR experiment. Once again, the addition of proline to the sample buffer appears to have alleviated the problem of aggregation. Under these conditions, structural characterization of ilvM will be feasible.

In the case of ilvN, gel filtration studies (vide supra) have shown that the protein most likely exists as an oligomer (dimer?) in phosphate buffer at pH 7.0. This can also be observed in the one-dimensional NMR spectrum of ilvN (Fig. 8A), in which one can observe broadened resonance lines for the protein. A significant line narrowing is observed when valine is added to the protein sample (Fig. 8B). This is a very clear indication of a specific interaction between valine and ilvN, causing the protein to undergo a transition from an oligomer to a monomer.

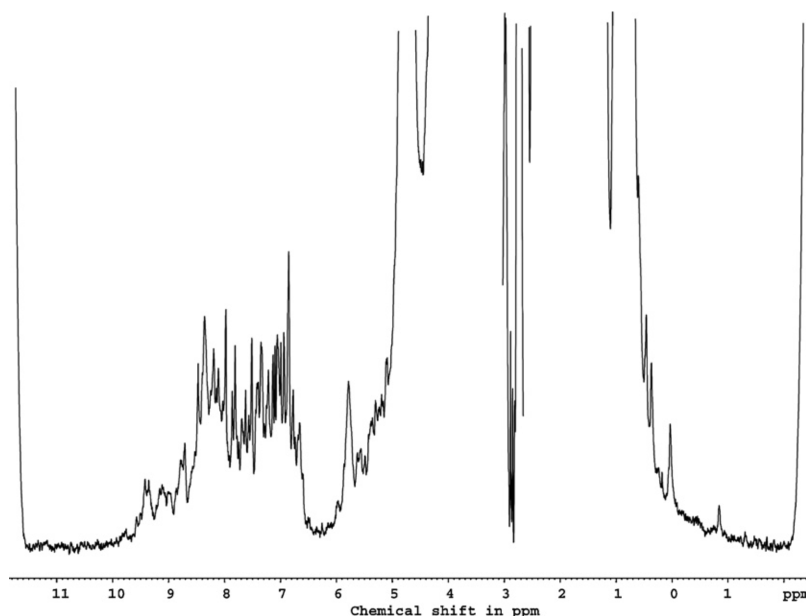

Fig. 7. Proton one-dimensional NMR spectrum of ilvM, the regulatory subunit of AHAS II. The spectrum shows well-resolved resonances. The spectrum has been acquired on a sample whose buffer conditions have been adjusted to include protein stabilizers such as arginine, glutamate and proline. The spectrum shows clear resolved resonances, indicating that this protein is amenable for structural studies after careful sample preparation.

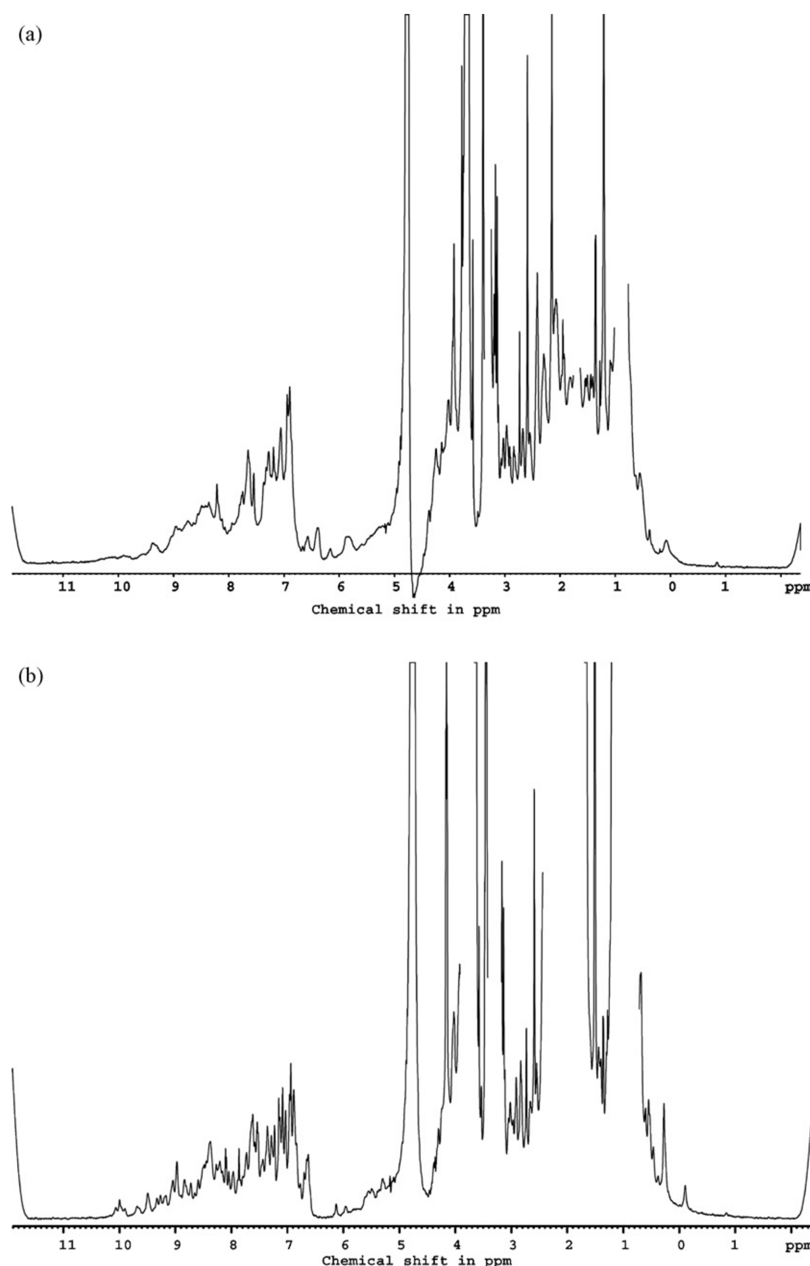

Fig. 8. (A) Proton one-dimensional NMR spectrum of ilvN, the regulatory domain of AHAS I. The spectrum was acquired on a sample prepared in phosphate buffer without adjuvants. The spectrum shows broadened resonances, indicative of the fact that the predominant molecular species is a high molecular weight one. (B) Proton one-dimensional NMR spectrum of ilvN in the presence of 5 mM valine. The line narrowing observed in the presence of valine (cf. (A)), indicates that valine specifically interacts with ilvN and most probably causes dissociation of the oligomer (dimer?) into a monomer.

4. Conclusions

The interaction between the regulatory subunit and the catalytic subunit of AHAS I from *E. coli* has been explored using a novel biochemical strategy. Our approach has been validated by the observation that the reconstituted enzyme is catalytically active. Indeed it must be noted that the ilvBα and ilvBγ complex should in principle be minimally required for formation of the active site. As we have shown, reconstituted ilvBα and ilvBγ are sufficient to catalyze the formation of acetolactate. Addition of ilvBβ only marginally increases the activity. Addition of ilvN to all three domains of ilvB increases the activity of the enzyme several fold. Thus we are confident that any structural information obtained on the interactions under these conditions could be related to a structural model using the structure of the holoenzyme as a template. Towards this end we have used NMR spectroscopy to address the structure of the FAD binding domain of ilvB and to probe the interactions of the ilvBα and ilvBβ domains of ilvB with ilvN. We surmise that interaction of ilvN with ilvBβ causes movement of this domain into the active site pocket, thereby enabling the FAD molecule to provide additional steric factors that enhances enzyme activity [5,8]. Efforts are underway to determine high-resolution structures of ilvN and ilvM. The preliminary results presented on the solution properties of these two proteins strongly suggest that these molecules will be amenable to structural characterization. In the case of ilvN, it is interesting to note the effect of valine on the solution properties. It is tempting to speculate that the regulatory subunits may undergo a dimer to monomer transition in the presence of valine as a mechanism for regulation. The structure if ilvH [4] shows that it too is a dimer. Furthermore, the N-terminal

region of ilvH has a high degree of similarity with ilvN. While the results presented here are preliminary, a more rigorous characterization in terms of structure and interaction will hopefully provide an insight into the mechanism of regulation of this important enzyme.

Acknowledgements

The authors would like to thank the Department of Biotechnology, Government of India for project funding. NMK is the recipient of a Government of India CSIR Senior Research Fellowship. The NMR spectroscopy and Mass spectrometric facilities are funded by the Department of Biotechnology and Department of Science and Technology, Government of India. SPS thanks the MHRD, Government of India for travel funds to attend the 7th International Conference on Mechanisms and Physiology of Thiamine.

References

[1] H.E. Umbarger, in: F.C. Neidhardt (Ed.), *Escherichia coli* and *Salmonella typhimurium*: Cellular and Molecular Biology, vol. 1, American Society for Microbiology, Washington, DC, 1987, pp. 352–367.
[2] S. Mendel, V. Vinogradov, M. Vyazmensky, D.M. Chipman, Z. Barak, J. Mol. Biol. 325 (2003) 275–284.
[3] S.S. Pang, R.G. Duggleby, L.W. Guddat, J. Mol. Biol. 317 (2002) 249–262.
[4] A. Kaplun, M. Vyazmensky, Y. Zherdev, I. Belenky, A. Slutzker, S. Mendel, Z. Barak, D.M. Chipman, B. Shannan, J. Mol. Biol. 357 (2006) 951–963.
[5] A. Mitra, S.P. Sarma, Biochemistry 47 (2008) 1518–1531.
[6] A. Mitra, K.S. Chakrabarti, M.S. Shahul Hameed, K.V. Srinivas, G.S. Kumar, S.P. Sarma, Protein Expr. Purif. 41 (2005) 84–97.
[7] C.M. Hill, S.S. Pang, R.G. Duggleby, Biochem. J. 327 (1997) 891–898.
[8] A. Mitra, PhD Thesis, 2008.