ELSEVIER

Journal of Chromatography B, 743 (2000) 225–229

JOURNAL OF CHROMATOGRAPHY B

www.elsevier.com/locate/chromb

Determination of the dissociation constant of valine from acetohydroxy acid synthase by equilibrium partition in an aqueous two-phase system

S. Engel[a], M. Vyazmensky[b], Z. Barak[b], D.M. Chipman[b], J.C. Merchuk[a,*]

[a]*Department of Chemical Engineering and Unit of Biotechnology, Ben-Gurion University of the Negev, Beer Sheva, Israel*
[b]*Department of Life Sciences, Ben-Gurion University of the Negev, Beer Sheva, Israel*

Abstract

An aqueous polyethylene glycol/salt two-phase system was used to estimate the dissociation constant, $K_{dis,}$ of the *Escherichia coli* isoenzyme AHAS III regulatory subunit, IlvH protein, from the feedback inhibitor valine. The amounts of the bound and free radioactive valine in the system were determined. A Scatchard plot of the data revealed a 1:1 valine–protein binding ratio and K_{dis} of 133±14 μM. The protein did not bind leucine, and the ilvH protein isolated from a valine resistant mutant showed no valine binding. This method is very simple, rapid and requires only a small amounts of protein compared to the presently used equilibrium dialysis method. © 2000 Elsevier Science B.V. All rights reserved.

Keywords: Aqueous two-phase systems; Dissociation constant; Partition coefficient; Valine; Acetohydroxy acid synthase; Enzymes

1. Introduction

Acetohydroxy acid synthase (AHAS) is the enzyme that catalyzes the first common step in the biosynthesis of the branched-chain amino acids in plants, yeast and bacteria [1]. AHAS isoenzyme III in *Escherichia coli* is composed of the large (62 kDa), IlvI catalytic subunit and the small (17 kDa), IlvH regulatory subunit (IlvH protein) [2]. The enzyme is reversibly inhibited by one of the pathway end-products, valine. The enzymatic activity of the catalytic subunit alone is resistant to valine, but valine sensitivity is restored upon the reconstitution of the holoenzyme [3]. It has been shown that valine binds to the regulatory IlvH protein [4]. There are mutant IlvH proteins that do not confer valine sensitivity on the enzyme upon reconstitution [4,5]. The comparison between the valine dissociation constant, K_{dis}, for the wild type IlvH protein and the valine resistant mutants can provide insight into structure–function relationships in the enzyme.

In the present work we consider the use of an equilibrium partition method [6,7] in an aqueous two-phase system (ATPS) to evaluate K_{dis} of valine from IlvH protein and the binding stoichiometry.

ATPS conditions were chosen, for which IlvH protein is located solely in the lower salt phase, whereas valine is distributed almost equally between the phases. The binding of valine to the protein shifts the observed partition of labeled valine between the phases. The amounts of the bound and free radioactive valine in the system were determined for

*Corresponding author.

0378-4347/00/$ – see front matter © 2000 Elsevier Science B.V. All rights reserved.
PII: S0378-4347(00)00050-5

different concentrations of valine and the protein and a Scatchard plot of the data [8] was used to obtain values for K_{dis} and the binding stoichiometry. Labeled leucine was used to demonstrate specificity of the valine–IlvH protein interaction.

2. Experimental

2.1. Materials

Polyethylene glycol (PEG) with a molecular mass of 4000 was obtained from Fluka (Buchs, Switzerland). L-[U-^{14}C]-valine and L-[4,5-^{3}H]-leucine were obtained from Amersham (UK). All other chemicals were of analytical grade.

2.2. Purification of AHAS III small subunit, IlvH

The wild type IlvH protein was purified from *Escherichia coli* XLMRF/pUH [9]. The Gly14Asp mutant IlvH protein was purified from *E. coli* BUM1 carrying the plasmid with a mutated gene *ilvH* [4]. The purification was carried out according to the method described previously [4].

2.3. Phase partition technique

The recombinant IlvH protein is soluble at a high Mg^{2+} ion concentration [4]. Considering this, $MgSO_4$ was chosen as constituent of the salt phase in a PEG/salt ATPS. The system prepared from stock solutions of PEG-4000 (60%, w/w) and $MgSO_4$ (23%, w/w) was equilibrated at 20°C. The pH of the stock solutions was adjusted with H_2SO_4 or KOH. The system PEG-4000 (12%, w/w)–$MgSO_4$ (7.5%, w/w) at pH 7.0 was chosen for the partitioning experiments. In this system the partition coefficient *K*, the concentration in the top PEG-rich phase divided by the concentration in the bottom salt rich phase, is 0.05 for the IlvH protein and 0.68 for valine. A constant amount of the protein and varying amounts of stock valine solution containing L-[U-^{14}C]-valine ($1\cdot10^6$ cpm/μmol), or alternatively, a constant amount of radioactive valine solution and varying amounts of the protein, were present in the assay tubes. To confirm specificity of the valine–IlvH protein interaction a control experiment was run with a leucine solution containing L-[4,5-^{3}H]-leucine ($25\cdot10^6$ cpm/μmol). The total system weight was 1 g. Equilibration of the system was carried out for 5 min at room temperature under gentle constant vortex agitation. The mixture was then centrifuged (300 *g*, 5 min) to complete separation of the two phases. The phase volume ratio was measured in a capillary tube.

2.4. Analyses

Aliquots of top phase (20 μl) were analyzed for total radioactivity in UltimaGold LSC cocktail (Packard Instrument, Meriden, CT, USA) in a Packard 1666TR TriCarb liquid scintillation counter.

Protein concentration was determined by the dye-binding method of Bradford [10], with bovine serum albumin as standard.

2.5. Calculations

The model used for valine binding parameters determination is shown in Fig. 1. In this system the partition coefficients of both IlvH protein and IlvH protein complex with valine are so low that their concentrations in the upper phase can be neglected.

From the measured concentration of valine in the upper phase, $[Val]_u$, we can calculate the concentration of free valine in the lower phase, $[Val]_L$, since $[Val]_L = [Val]_u/K$, where *K* is partition coefficient of valine. From the known total amounts of valine and IlvH protein added to the system, and the volumes of

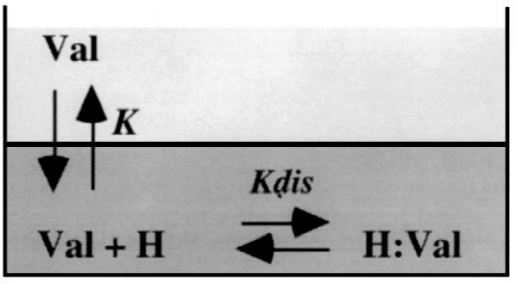

Fig. 1. Equilibria in the system. The IlvH protein (H) is located solely in the salt phase, whereas valine (Val) is distributed almost equally between the phases (the partition coefficient of valine, *K*, is 0.68). IlvH:Val is a IlvH protein complex with valine. K_{dis} is a dissociation constant of valine from IlvH protein.

the phases, we can calculate both free IlvH protein and bound valine concentrations in the lower phase. The binding parameters K_{dis} and n were obtained using the Scatchard equation [8]:

$$\text{Bound/Free} = (n \cdot P_t - \text{Bound})/K_{dis}$$

where K_{dis} is the dissociation constant, n is the number of identical non-interacting binding sites per protein molecule and P_t is the total protein concentration.

All calculations were performed with the average of data obtained from two independent experiments. The experimental values measured differed by less than 10%.

3. Results and discussion

3.1. Determination of K_{dis} of valine and the wild type IlvH protein

Fig. 2 demonstrates the specificity of the valine–IlvH protein interaction. The presence of IlvH protein in the system had no effect on the partition coefficient of the radioactive leucine. There is no bound leucine in the system (A). By contrast, valine binds to IlvH protein in the lower phase, thus its apparent partition changes (B). A Scatchard plot of the binding of valine to wild type IlvH protein fitted by linear regression is shown in Fig. 3. The intercept on the abscissa indicates a 1:1 binding stoichiometry of valine to IlvH protein at saturation ($n = \text{Bound}/P_t$ is close to 1). The apparent dissociation constant, K_{dis}, is 133 ± 14 μM. The value of K_{dis} obtained previously by Vyazmensky et al. [4] using equilibrium dialysis method was 190 ± 40 μM, but the binding stoichiometry of valine was about half of the expected.

Fig. 4 shows, that the dissociation constant is not changed over a range of concentrations of wild type IlvH protein, suggesting that a 1:1 complex is indeed formed.

Since the dissolving of the recombinant IlvH proteins requires the presence of a high Mg^{2+} ion concentration, the equilibrium dialysis experiments mentioned above were performed with the high Mg^{2+} content [4]. The lower phase of the aqueous

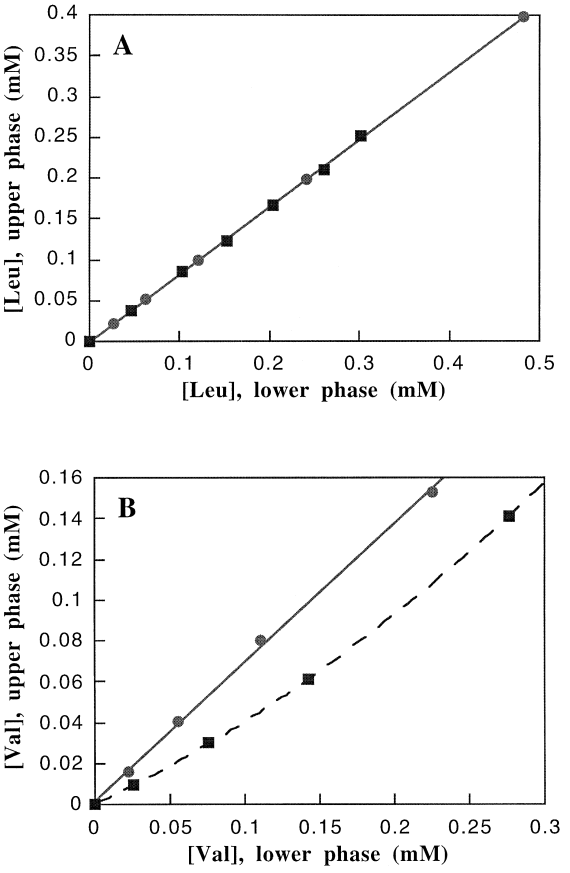

Fig. 2. (A) Partition behavior of labeled leucine. A slope of the plot defines the partition coefficient, K, of leucine in the system. Circles: the partition of leucine alone, $K = 0.83$; squares: partition of leucine with 0.151 mM (1 mg) of IlvH protein (wild type) presents in the system. (B) Partition behavior of labeled valine. Circles: the partition of valine alone, $K = 0.68$; squares: partition of valine with 0.151 mM of IlvH protein (wild type) present in the system.

two-phase system also contains high (0.8 M) Mg^{2+} concentration. It appears, that high salt concentration does not prevent valine binding to the IlvH protein. The binding parameters at low salt concentration (physiological conditions) could be estimated by an extrapolation, if the functionality between K_{dis} values and the salt concentrations in the system was known. However, the location of the used ATPS on the equilibrium plane was close to the binodal curve. Therefore, the only possibility of exploring the functionality between K_{dis} and the salt concentration was to work on systems with increasing salt content

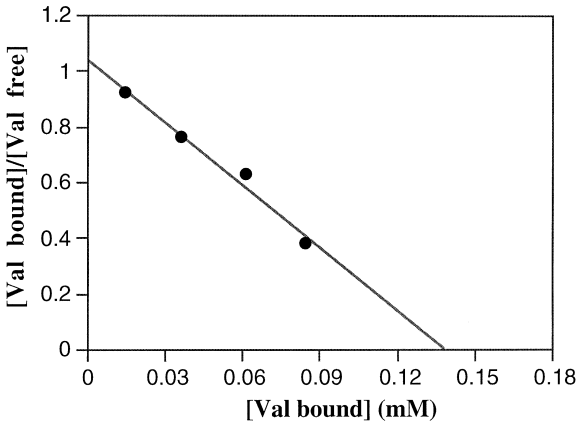

Fig. 3. Scatchard plot of the binding of valine to wild type IlvH protein. The protein concentration in the system was 0.151 m*M* (1 mg in the 1 g system). The binding parameters calculated by linear regression were: $K_{dis}=133\pm14$ μ*M*; $n=0.93$. Correlation coefficient of the fit was 0.989.

(further from the binodal curve). This was done, but no clear correlation between K_{dis} and the salt concentration in the system was found, which prevented the extrapolation of K_{dis} to physiological conditions.

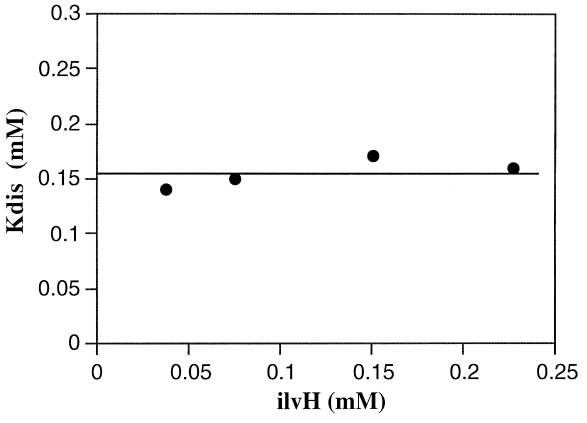

Fig. 4. Plot of dissociation constant K_{dis} as a function of protein concentration in the system. The concentration of valine was fixed at 0.02 m*M*. K_{dis} values were calculated from $K_{dis}=[Val]_L\cdot[H]_L/[H:Val]_L$, where $[Val]_L$, $[H]_L$ and $[H:Val]_L$ are the lower phase concentrations of free valine, free IlvH protein and IlvH protein complex with valine, respectively. The K_{dis} value obtained by this calculation was 155 μ*M*, standard deviation 12 μ*M*.

3.2. Determination of K_{dis} of valine from the mutant IlvH protein Gly14Asp

The same ATPS method and conditions were applied to the mutant Gly14Asp IlvH protein, obtained from a spontaneous valine-insensitive mutant [5]. Within the limits of sensitivity of the system, no valine binding to the mutant protein could be detected. This is consistent with the results obtained by the equilibrium dialysis method mentioned previously [4], and demonstrates that the observed effect of the IlvH protein on the distribution of the labeled amino acid is not a non-specific protein effect. This was also demonstrated by the control experiment, in which leucine was used in place of valine; leucine is a very weak effector of AHAS III.

4. Conclusions

Comparative studies of wild type and mutated proteins are widely used for investigation of structure–function relationships in a protein (enzyme). Equilibrium partitioning appears to be a very suitable method for this kind of studies, in which binding sites for small molecules on a protein molecule are investigated and physiological conditions are not of critical importance.

In the present work we elaborated a method for comparative measurement of K_{dis} of valine from the AHAS small subunit protein by use of equilibrium partitioning in the PEG/salt aqueous two-phase system. The high salt concentration in the system does not prevent the binding of the inhibitor valine to IlvH protein, and does not affect the specificity of the interaction.

In contrast to the equilibrium dialysis, this method is experimentally simple and requires neither special instrumentation nor long equilibration times. Due to intimate phase contact and the absence of mechanical barriers to diffusion, the equilibrium conditions in the system are reached very fast. Moreover, this method requires only small amount of protein and ligand, and is amenable to scaling down. This is extremely important for mutant proteins, where available amounts may be quite low.

Acknowledgements

The help of Paulina Wainer in the work is gratefully acknowledged. This work was partially supported by grant 243/98 from the Israel Science Foundation.

References

[1] H.E. Umbarger, in: F.C. Neidhardt, J.L. Ingraham, K. Low, B. Magasanik, M. Schaechter, H.E. Umbarger (Eds.), *Escherichia coli* and *Salmonella typhimurium*, Cellular and Molecular Biology, Vol. 1, American Society for Microbiology, Washington, DC, 1987, p. 352.
[2] C.H. Squires, M. DeFelice, J. Devereux, J.M. Calvo, Nucleic Acids Res. 11 (1983) 5299.
[3] O. Weinstock, C. Sella, Z. Barak, D.M. Chipman, J. Bacteriol. 174 (1992) 5560.
[4] M. Vyazmensky, C. Sella, Z. Barak, D.M. Chipman, Biochemistry 35 (1996) 10339.
[5] C. Sella, The Role of the Small Subunit of Acetohydroxy acid Synthase III in Feedback Inhibition by Valine and in Subunit Interaction, Ph.D. Thesis, Ben-Gurion University of the Negev, Beer Sheva, 1996.
[6] C. Gray, J. Chamberlin, Anal. Biochem. 41 (1971) 83.
[7] P.A. Albertsson, Partition of Cell Particles and Macromolecules, Wiley, New York, 1986.
[8] G. Scatchard, Ann. NY Acad. Sci. 51 (1949) 660.
[9] M. Vyazmensky, T. Elkayam, D.M. Chipman, Z. Barak, Methods Enzymol. Branched Chain Amino Acids, part B, in press.
[10] M. Bradford, Anal. Biochem. 72 (1976) 248.