Characterization of Amino Acid tRNA Ligases using the Analytical Ultracentrifuge

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Akademisk avhandling för filosofie doktorsexamen i kemi med inriktning mot biokemi, som med tillstånd från Naturvetenskapliga fakulteten kommer att offentligt försvaras tisdagen den 29, september, 2015 kl. 13.00 i Hörsal Ragnar Sandberg, Medicinaregatan 7A, Göteborg.

Characterization of Amino Acid tRNA Ligases using the Analytical Ultracentrifuge

Doctoral thesis

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Cover

Images of various analytical ultracentrifuges from Center for Analytical Ultracentrifugation of Macromolecular Assemblies, CAUMA, Virgil Schirf and Karel L. Planken, December 2008.

Image A shows an early (oil turbine) analytical ultracentrifuge as employed by T. Svedberg (with kind permission of the Royal Society of Chemistry).

Image B shows the Specialized Instruments Corporation (Spinco/Beckman) Model E analytical ultracentrifuge.

Image C shows the AUC facility of our Van 't Hoff Laboratory in 2008.
To date, the Beckman CoulterTM OptimaTM XL-A (on the right of image C) and XL-I (on the left of image C) analytical ultracentrifuges are employed for sedimentation experiments.

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Online version

ISBN: 978-91-628-9518-1
Available at http://hdl.handle.net/2077/39312

Print version

ISBN: 978-91-628-9517-4
Printed and bound by Aidla Trading AB, 2015.

Better late than never.

The Yeoman´s Prologue and Tale, Canterbury Tales, circa 1386:

For bet than never is late. Which means that: Better than never is late.

To arrive or do something later than expected isn´t good, but it is better than not at all.

This thesis is dedicated to my children

Johan Mats Lisa

and my brother

Owe

ABSTRACT.

Quaternary structures of amino acid tRNA ligases/synthetases (aaRS) in the native as well as in denatured forms were examined by molecular weight determinations (paper I-IV). Analytical ultracentrifugation, gel electrophoresis, gel chromatography were used in these investigations. The aaRSs were obtained from bacteria and yeast: LysRS and ValRS from S. cerevisiae, AspRS, LysRS and SerRS from E. coli, AsnRS, LysRS, SerRS and ValRS from Bacillus stearothermophilus. The quaternary structures of ValRSs from S. cerevisiae and B. stearothermophilus are both monomeric (the α-type), whereas all the other aaRSs are homodimers (the $\alpha 2$-type). Two of the aaRS-enzymes have also been crystallized. Both of them are LysRS and their structures were examined with X-ray crystallography. LysRS from S. cerevisiae with a resolution of 5 to 6 A in all directions, and from B. stearothermophilus LysRS with a resolution of 8 A.

In Paper V the enzyme HMG-CoA lyase was investigated. The activity of this enzyme is found in Rhodospirillum rubrum cells grown anaerobically in the light with leucine as the carbon source. A 1.2 kb long DNA segment from R. rubrum has been sequenced and includes the first identified gene for a putative 3-hydroxy-3-methylglutaryl-CoA (HMG-CoA) lyase, termed hmgL, from a photosynthetic organism.

A parallel project concerned the characterization of a DNA homeobox (HD), where it was shown that the T7 promoter sequence lacked an important guanine for the transcription of this gene.

The analytical ultracentrifugation method as described in this thesis played an important role already when the first protein structures were characterized, and the interest has increased dramatically during the last ten years partly due to automation. I hope that my early work on the application of analytical ultracentrifugation to tRNA ligases/synthetases (aaRSs) also helped to inspire these exciting developments.

LIST OF PAPERS.

This thesis is presented as a summary based on the following papers referred to in the text by their Roman numerals:

I. Rymo, L., Lundvik, L., and Lagerkvist, U. Subunit Structure and Binding Properties of Three Amino Acid Transfer Ribonucleic Acid Ligases. J. Biol. Chem. 247, 3888-3899 (1972).

II. Lundvik L., Lustig, F. and Rymo, L. Properties of the Sulfhydryl Groups of Three Amino Acid: Transfer RNA Ligases. Acta Chem. Scand. B 31 95-101 (1977).

III. Åkesson, B. and Lundvik, L. Simultaneous Purification and Some Properties of Aspartate: tRNA Ligase and Seven Other Amino-acid: tRNA Ligases from *Escherichia coli.* Eur. J. Biochem. 83, 29-36 (1978).

IV. Samuelsson, T. and Lundvik, L. Purification and Some Properties of Asparagine, Lysine, Serine, and Valine: tRNA Ligases from *Bacillus stearothermophilus.* J. Biol. Chem. 253, 7033-7039 (1978).

V. Baltscheffsky, M., Brosche, M., Hultman, Th., Lundvik, L., Nyren, P., Sakai-More, Y., Severin, A., and Strid, Å. A 3-Hydroxy-3-Methylglutaryl-CoA Lyase Gene In the Photosynthetic Bacterium *Rhodospirillum rubrum*. Biochim. Biophys. Acta 1337, 113-122 (1997).

Other paper not included in this thesis:

Jontell, M., Linde, A., and Lundvik, L. Comparative Studies of Phosphoprotein Preparations from Rat Incisor Dentin. Prep. Biochem. 10(3), 235-253 (1980).

Svensk sammanfattning av innehållet i denna avhandling med titeln “Characterization of Amino Acid tRNA Ligases using the Analytical Ultracentrifuge”.

I. Subunit Structure and Binding Properties of Three Amino Acid Transfer Ribonucleic Acid Ligases.

Karakterisering av tre aminosyra tRNA ligaser: E^{Lys} från jäst och E. coli samt E^{Val} från jäst.

Med analytisk ultracentrifug kunde dessa bestämmas med avseende på molvikter både nativt och denaturerat bestående av två identiska subenheter för E^{Lys} från jäst och E. coli men endast en enhet för E^{Val} från jäst. Vid samtliga bestämningar användes jämviktscentrifugering med avseende på sedimentation.

Renheten hos dessa framställda enzymer kontrollerades med hastighetskörning för sedimentationen som påvisades med en så kallad Schlieren topp. Denna var helt symmetrisk utan bidrag från några fragment av dessa enzymer.

II. Properties of the Sulfhydryl Groups of Three Amino Acid: Transfer RNA Ligases.

Här kunde konstateras att de tidigare studerade aminosyra tRNA ligaserna från jäst och E.coli uppvisade konformationsförändring som var påvisbar med hjälp av CD vid våglängden 220 nm där de studerade liganderna saknade bidrag avseende α-helix.

Tre system användes för detta:

1. Enzym med motsvarande aminosyra.

2. Enzym med ATP, Mg^{2+} och aminosyra.

3. Enzym med motsvarande tRNA och Mg^{2+} .

Med avseende på α-helix innehåll hos dessa tre enzymer så visade sig system 2 ge 11 % mer α-helix för E^{Lys} från jäst och 26,6 % mer α-helix för E^{Val} från jäst.

System 3 visade mindre α-helix med 11 % för E^{Lys} från jäst och 8,3 % för E^{Lys} från E.coli.

Detta noterades inte i publikationen men finns med i avhandlingen.

III. Simultaneous Purification and Some Properties of Aspartate: tRNA Ligase and Seven Other Amino-acid: tRNA Ligases from *Escherichia coli.*

På samma sätt som vid publikation I studerades E^{Asp} och E^{Ser} från E. coli. Med analytisk ultracentrifug och jämviktscentrifugering av sedimentationen kunde både nativt och denaturerat enzym ge upplysning om förekomst av subenheter. Det visade sig vara två identiska enheter i dessa båda enzym. Storleken av dessa bekräftades även med SDS-PAGE.

Det nativa enzymets storlek kunde också erhållas med gelfiltrering.

Renheten av provet innehållande enzymet kontrollerades dels med analytisk ultracentrifug och sedimentationshastighet som gav en symmetrisk Schlieren topp. Dels också med PAGE som ett enda band på gelen för E^{Asp}.

IV. Purification and Some Properties of Asparagine, Lysine, Serine, and Valine: tRNA Ligases from *Bacillus stearothermophilus.*

Här användes en bakterie som är mycket värmestabil nämligen *Bacillus stearothermophilus.*

Fyra aminosyra tRNA ligaser studerades såsom: E^{Asn}, E^{Lys}, E^{Ser} och E^{Val}.

Resultatet av dessa studier med avseende på förekomst av subenheter gav följande:

Tre av dessa enzymer såsom: E^{Asn}, E^{Lys} och E^{Ser} hade två identiska subenheter men E^{Val} bestod av en enhet som redovisas i avhandlingen men inte i publikationen.

Värmestudier i temperatur intervallet 50 - 70° C visade att bindnings-ställen för ATP och aminosyra på enzymet påverkades mer än stället för bindandet av tRNA. Detta tyder på att det senare är mera beläget på utsidan av enzymet medan de andra binder i det inre av detta.

V. A 3-Hydroxy-3-Methylglutaryl-CoA Lyase Gene In the Photosynthetic Bacterium *Rhodospirillum rubrum*.

Ett enzym från en fotosyntetisk bakterie *Rhodospirillum rubrum* studerades. Först med DNA sekvensering av hmgL genen för detta enzym HMGL som erhölls från en plasmid pÅS6 som är genen hmgL insatt i vektorn pET3a. Enzymet erhölls efter transformation av denna plasmid i E. coli bakterie som användes för överuttryck genom induktion med IPTG. Diverse olika expressions-system användes och slutligen erhölls en ny plasmid pCYB1 / hmgL som transformerades in i E.coli.

Det tidigare erhållna enzymet HMGL kontrollerades med avseende på enzymaktivitet med olika ”kolkällor” som substrat och NADH syntes som påvisades i spektrofotometer. Detta som bevis på att acetoacetat som är en så kallad ketonkropp bildats.

CONTRIBUTION REPORT

Paper I, Paper II, Paper III, Paper IV.

I performed all the Mr determinations for the different proteins with analytical ultracentrifugations under native conditions as well as under denatured conditions. The analytical methods were sedimentation velocity with Schlieren optics and sedimentation equilibrium with Interference optics. Moreover, I used gelfiltrations as well as SDS-PAGE for confirmation of the Mr results. I was not involved in the Appendix belonging to Paper I.

Paper II

I performed binding studies with different ligands for studies of conformational changes in the enzymes with Circular Dichroism in the Cary 60 spectropolarimeter as the analytical instrument.

Paper V

I performed manually the DNA sequencing of the gene for 3-hydroxy-3-methylglutaryl-CoA (HMG-CoA) lyase, termed hmgL (from *R. rubrum*), with an ALFexpress DNA sequencer. The plasmid with this hmgL in the vector pET3a was first transformed into *E. coli* BL21(DE3)pLysS and the hmgL was finally in the vector pCYB1 to be transformed into *E. coli* K12 for the production of HMG-CoA lyase (HMGL). I performed measurement of the enzyme HMGL activity to study the formation of acetyl-CoA and the ketone body acetoacetate from HMG-CoA.

ABBREVIATIONS.

A	aminoacyl site (ribosome)
aa	amino acid
aa-AMP	aminoacyl adenylate
aaRS	aminoacyl-tRNA synthetase (EC 6. 1. 1) or amino acid tRNA ligase
aa-tRNA	aminoacyl-tRNA
AMP	adenosine monophosphate
ATP	adenosine triphosphate
BD	benzoylated DEAE
CD	circular dichroism
CPC	cetyl pyridinium chloride
CTL	cytotoxic T lymphocyte cell
DEAE	di-ethyl-amino-ethyl
DHU	di-hydro-uridine (= UH_2)
DNA	deoxyribo-nucleic acid
DTNB	5,5´-di-thiobis (2-nitro-benzoic acid) (= Nbs_2)
DTT	di-thio-threitol
E	exit site (ribosome)
EDTA	ethylene-diamine-tetra-acetate
fMet	formyl methionine
GDP	guanosine diphosphate
GSH	glutathione
GTP	guanosine triphosphate
GuHCl	guanidine-HCl
HD	homeodomain
HG	3-hydroxyglutarate
HMG-CoA	3-hydroxy-3-methylglutaryl-CoA
hmgL	HMG-CoA lyase gene
IF	initiation factor
IPTG	Isopropyl β-D-1-thiogalactopyranoside
Mr	molecular weight (reduced weight average)
Mw	molecular weight
mRNA	messenger ribonucleic acid
NMR	nucleo magnetic resonance
P	peptidyl site (ribosome)
PAGE	poly-acrylamide gel-electrophoresis
PCR	polymerase chain reaction
P_i	phosphate (inorganic)
PP_i	pyrophosphate (inorganic)
pI	isoelectric point
PMSF	phenyl methyl sulfonyl fluoride
RE	restriction enzyme
RF	release factor
RNA	ribonucleic acid

RNAP	RNA polymerase
rpm	revolution per minute
rRNA	ribosomal ribonucleic acid
RS^-	protein (enzyme) with thiol group
SDS	sodium-dodecyl-sulfate
tRNA	transfer ribonucleic acid
$tRNA^{aa}$	amino acid specific tRNA
Tu	transfer (elongation) factor (unstable) (= EF-Tu)
Ts	transfer (elongation) factor (stable) (= EF-Ts)
TψC	ψ= pseudouridine

KEYWORDS:

amino acid tRNA ligase; analytical ultracentrifugation (AUC); antennapedia; Antp gene; binding site; circular dichroism; conformational change; HMG-CoA lyase; homeodomain; ketone bodies; molecular weight; pAop2CS ; quaternary structure; temperature inhibition; thiol group; tRNA; X-ray crystallography.

CONTENTS.

1. AIMS OF THIS THESIS

The first four papers in this thesis (I-IV) aim at the characterization of structure and conformational changes of amino acid tRNA ligases, a class of enzymes that is still a focus of todays research (see Figs. 1 and 2).

The goal of the studies of the enzyme HMGL was to receive the possibility to get a more human-like alternative for the production of reserve energy when this was needed for people from under–developed countries.

The parallel project with characterization of the transcription factor AntpHD aimed at getting better knowledge for the development of different cells finally producing new organic parts in the whole body.

Fig. 1. Published Items in Each Year for "Amino Acid tRNA Ligases".

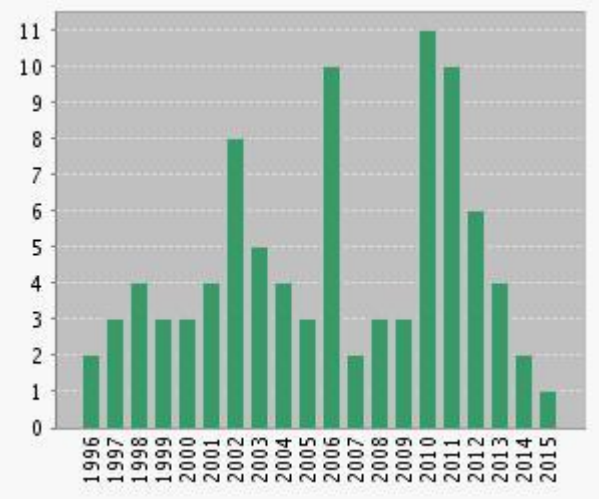
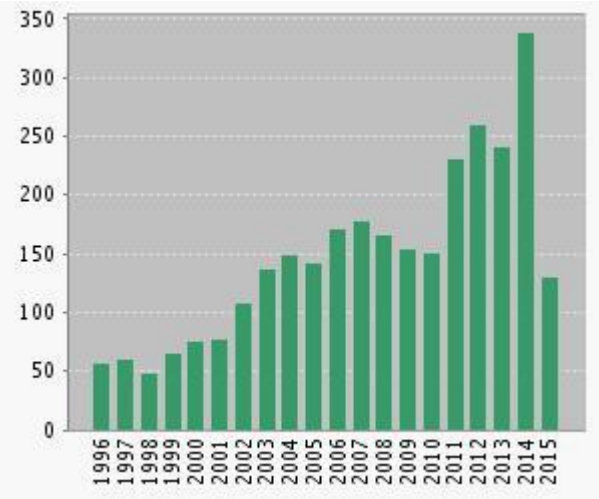


Fig. 2. Citations in Each Year for "Amino Acid tRNA Ligases".



2. INTRODUCTION

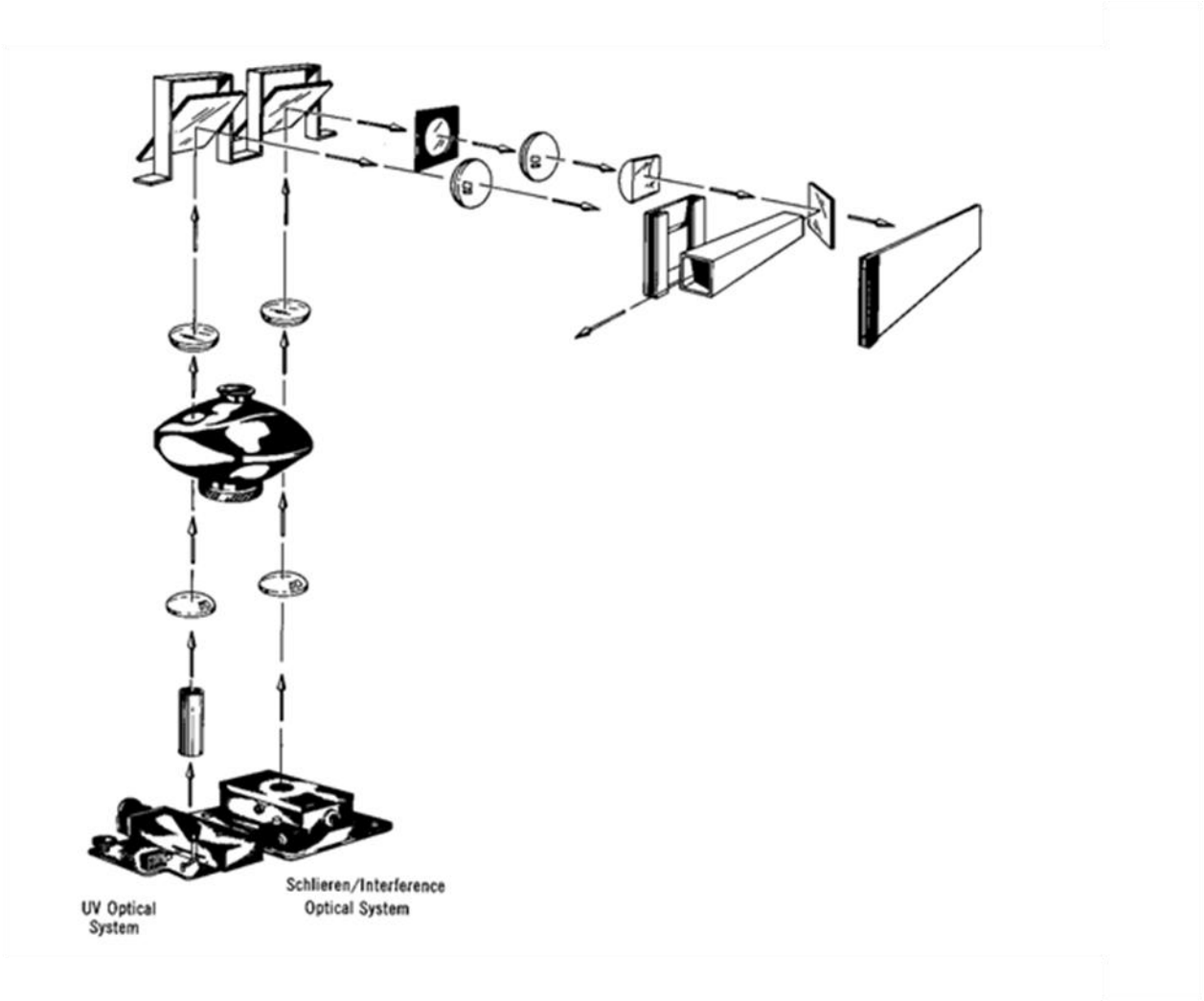
2.1. Interactions between proteins and other molecules

The immune response is mediated by the interaction of the immunoglobulin (antibody) protein with an antigen molecule. Metabolic processes are regulated by the binding of small molecules to enzymes. Muscle contraction is mediated by interaction of calcium ions with regulatory proteins that in turn interact with the contractile proteins to initiate contractile work. Understanding the nature of these interactions is central to understanding the chemical and physical processes that constitute life. Many main interests are in the application of physical methods to the study of dynamically reversible protein-protein interactions (PPI) and protein-nucleic acid interactions that are relevant to physiological function in normal and diseased states. Many interactions peculiar to disease states can be targets for drug therapy. These antibody molecules can be used as vehicles for the targeted delivery of therapeutic agents to kill breast cancer cells. The engineered antibody proteins are produced by using techniques of molecular biology to produce antibody molecules with the desired reactivity and properties. A very important step in designing protein molecules is the assessment of their properties in solution and their strength of binding to unique molecules found on the surface of breast cancer cells. The physical methods of analytical ultracentrifugation are being applied to the analysis of the strength of binding of the antibody molecules after changes are engineered into them. New highly sensitive techniques for the analytical ultracentrifuge have been developed and are being applied to these and other problems.

2.2. Historical reflection

The results from these examinations concerning molecular weights and quaternary structures of the proteins (Papers I – IV) were obtained by using the methods Sedimentation Velocity and Sedimentation Equilibrium. These analysis are still applied today with the current versions of the analytical ultracentrifuges. I had to do all optical corrections for the whole optical system with focusing of the many lenses involved in this (Fig. 3) before it was possible to begin with registration of analytical data for the proteins I examined and made calculations for from experimental results received from the work with the Spinco Model E analytical ultracentrifuge. The work was done between 1970 to 1980 when the use of this examination with Spinco Model E was very hard to handle without long-term experience before the results from experiments could be received with this instrument. Data was collected on film and manually interpreted. The structure of DNA and isolation of the polio virus are also linked to work on the Spinco[1] Model E.

Fig. 3. Spinco Model E optical parts.



2.3. DNA technologies

After a break I returned to research at University of Gothenburg in the mid 1990th but this time with slightly different objectives and the use of different technologies. The emphasis was now on DNA and the use of a wide variety of different techniques in gene technology The beginning of this research was concerned the enzyme HMGL which can produce ketone bodies essential for reserve energy. During starvation this missing of HMGL can be dangerous and is a deadly risk factor in certain under-developed areas in the world. The production and purification of this enzyme from bacteria was studied with the purpose of later use with humans (Paper V).

The interest in cell differentiation for many different cell organisms from bacteria to plant and also human beings[2] has been studied since long[3] . The origin for this was already in late 1980th presented from the group of Walter Gehring[4] in Basel Schwitzerland. The insect *Dosophila melanogaster* was used to receive the gene which resulted in the so called homeodomain (HD) which could bind to DNA and in this way produce different cell organelles[5,6,7,8].

When they made one mutant of this gene they got the result of *Antennapedia* (Fig. 4.) which means the change for legs instead of antennae[9] in this insect. The short name Antp stands for this and another mutation when one amino acid cystein (C) in position 39 of the sequence was changed to serine (S) resulted in a monomer of this protein instead of the dimer. This was given the complete name Antp(C39S) for this homeodomain.

Fig. 4. The mutation *Antennapedia.*

Retrieved from:”http//en.wikipedia.org/w/index.php?title=Antennapedia&oldid=566572503

3. HISTORICAL REFLECTIONS ON THE BIRTH OF MODERN BIOCHEMISTRY

3.1. Background

The living cell is the smallest part of all biological materia, such as plants and animals, which was first proposed by Schleiden and Schwann in 1839. The chemical basis of life in simple cells like bacteria and yeast is very much alike those in higher organisms. Cells in higher organisms can differentiate into cell types in different organs but they still keep their properties as single cells. This "vitalism" was for a long time thought to be some vital force outside the laws of chemistry. At first the most important group of macromolecules was believed to be the proteins because of the evidence that all enzymes are proteins. It was also found that the chromosomes contained proteins so the genes with high degree of specificity were thought to be proteins instead of the other chromosomal component, the nucleic acids. Nucleic acids were thought to be too small molecules in size and incapable of carrying sufficient genetic information which, as is now known, was wrong.

The chemical behaviour of small molecules of importance in life science, like the amino acids and nucleotides was first understood. The larger molecules, like proteins and nucleic acids, were difficult to study because they were much less stable and lost their activity during isolation. Before it was possible to get the detailed structure of a protein much work was needed to establish that the protein was chemically pure and biologically active. This demanded techniques for isolation, to reveal homogeneity of the product and to provide data on molecular size. To answer these questions regarding the physical-chemical properties of macromolecules in solution, topics like osmotic properties and the movement of macromolecules under electrical and centrifugal forces were investigated.
A striking contribution to the study of biological macromolecules was the development, in the 1920s, of centrifuges that rotated at high speed (ultracentrifuges) and that could cause rapid sedimentation of proteins and nucleic acids. Ultracentrifuges equipped with optical devices to observe exactly how fast the molecules sedimented were extremely valuable in obtaining data on the molecular weight of proteins and establishing the concept that proteins, like smaller biological molecules, are of discrete molecular weights and shapes. It was found that the sizes of proteins vary with molecular weights between 10,000 and 1,000,000.
The composition of proteins was also studied by means of chromatography. Since Fischer's[10] establishment that protein molecules are composed of amino acids linked by peptide bonds it was not until 1941 when Martin[11] and Synge developed separation methods (partition chromatography) it became a routine matter to separate quantitatively the 20 amino acids found in proteins. Sanger[12] used these methods to solve the primary structure of insulin, which was of great importance in the study of proteins to show that each type of protein has a specific composition and sequence of amino acids.
Another very important technique used in understanding macromolecules was X-ray crystallography. With this technique it was possible to exactly identify the three-

dimensional (3-D) arrangement of the atoms in molecules. Since the initial crystallization of inorganic molecules many important cell components have been crystallized which has resulted in resolved structures like the one of hemoglobin by Perutz[13] and myoglobin by Kendrew[14]. In 1951 Pauling[15] proposed a structure which he called the alpha-helix. It was a stereochemical arrangement of amino acids linked together by peptide bonds in helical configuration. This theory was also supported by Perutz's[16] work with synthetic polypeptide chains. By means of heavy atoms attached to protein molecules it was possible in 1953 to obtain correct structures from X-ray diffraction data. In 1959 this method helped Perutz and Kendrew to solve the structures of hemoglobin [13] and myoglobin[14] with their amino acid chains folded as α-helices in some regions and very irregularly in others. Because of Pauling's α-helix theory in 1951 it was possible to develop an elegant theory for the diffraction of helical molecules which finally helped Watson[17] and Crick to solve the DNA structure, a complementary double helix, in 1953. How DNA controls the sequence of amino acids in proteins is summarized by the formula called the central dogma. Exception to the central dogma is certain RNA viruses (<—).

Avery[18] and his co-workers reported in 1943 that the active genetic component was deoxyribonucleic acid (DNA). Now it is known that the chromosome with its genes are made of DNA in all prokaryotic and eukaryotic organisms. In viruses, genes consists either of DNA or of ribonucleic acid (RNA). In the cell the DNA double helix consists of two antiparallell and complementary polynucleotide strands. In the replication of DNA, the two strands of a double helix unwind and separate as new chains are synthesized. Each parent strand acts as a template for the formation of a new complementary strand Stryer[19].

The sugar unit in RNA is ribose whereas deoxyribose is found in DNA. Another difference is the base uracil (U) in RNA instead of thymine (T) in DNA. In RNA uracil form base pairs with adenine (A). Cytosine (C) is always paired with guanine (G). Cells contain three kinds of RNA: messenger RNA (mRNA), transfer RNA (tRNA), and ribosomal RNA (rRNA). All three types of RNA are synthesized according to instructions given by a DNA template. Transcription occurs at one of the unwinded DNA strands according to the sequence in different genes catalysed by RNA-polymerases.

Recent results from studies of one nucleotide guanosine tetraphosphate abbreviated as ppGpp[20] ("magic spot") concerning the role as control of the gene expression promoter - specifically by interacting with RNA polymerase (RNAP) without binding to DNA. Its mechanism of action with a binding site for ppGpp on *E. coli* RNAP has been identified by crosslinking, protease mapping, and analysis of mutant RNAPs that fail to respond to ppGpp. The binding site is at an interface of two RNAP subunits, ω and β´, and its position suggests an allosteric mechanism of action involving restriction of motion between two mobile RNAP modules. Identification of

the binding site allows prediction of bacterial species in which ppGpp exerts its effects by targeting RNAP. Genes responsible for ppGpp synthesis and degradation (the *relA* family) have been identified in almost all bacterial genomes examined, as well as in chloroplasts. The response to amino acid starvation referred to as the stringent response includes an extensive reprogramming of transcription. Binding of ppGpp to *E. coli* RNA polymerase in vitro and in vivo inhibits transcription from many promoters required for ribosome synthesis, activates transcription from a number of promoters for amino acid biosynthesis, and regulates a variety of additional promoters as well. Genome-wide approaches indicate that ppGpp regulates the levels of transcripts from several hundred genes involved in macromolecular biosynthesis pathways and a variety of stress responses, justifying the original name given to the modified nucleotide, "magic spot".

The language of the nucleic acids (nucleotides) must in some way be translated to the language of the proteins (amino acids) when the genetic information is expressed to a protein molecule. According to the adaptor hypothesis, (at least) 20 different adaptors (now known as tRNA) are needed, one (or more) each for the 20 different amino acids, to bring the information from the base sequence in RNA (now known as mRNA) to the growing polypeptide chain (at the ribosome). The base sequence in mRNA is read in triplettes (codons) by the complementary base sequence in tRNA (anticodon). The tRNA has a free 3'-end of bases (-C-C-A) where the amino acid specific for that tRNA is fixed.

Recent studies have begun to reveal the molecular mechanisms for quality control[21] concerning accuracy during the selection of aminoacyl-tRNAs on the ribosome and their base pairing with mRNA. The accuracy of tRNA selection is further enhanced in vivo by competition between ligases for their cognate tRNAs and in some cases by the recruitment of additional proteins that enhance binding. The principal reason that the inability to discriminate similar amino acids does not compomise the fidelity of translation is that the respective aminoacyl-tRNA ligases have proofreading activities. These activities have been found to operate at two levels: Most commonly, the activated noncognate aminoacyl-adenylate is hydrolyzed before transfer to tRNA can occur; less frequently, a noncognate aminoacyl-tRNA may be ligated that is then deacylated. The molecular mechanisms underlying these proofreading activities have recently been elucidated for isoleucyl-tRNA ligase. This enzyme contains two distinct catalytic sites that present a double sieve during substrate selection. The first sieve serves to exclude amino acids larger than isoleucine from the active site but is unable to exclude valine, and consequently valyl-AMP (adenosine monophosphate) is ligated.

The second sieve then acts by hydrolyzing valyl-AMP at a structurally distinct "editing" site. The proofreading activity of isoleucyl-tRNA ligase is dependent on specific sequences in cognate isoleucine tRNA species, which trigger the translocation of misactivated valine from the catalytic to the editing site, further enhancing the accuracy of isoleucyl-tRNA ligation by the enzyme. Isoleucyl-tRNA ligase provides a highly effective point of quality control, as seen from the observation that only about 1 in 3000 isoleucine codons are misread as valine during protein synthesis.

4. tRNA LIGASES AND AMINO ACID ACTIVATION

4.1. Description of players in translation

4.1.1. Over all reactions

The covalent linking of an amino acid (aa) to its specific tRNA is made in two steps. First the amino acid is bound to adenylic acid (AMP) by cleavage of adenosine triphosphate (ATP) catalyzed by the enzyme aminoacyl-tRNA ligase (aaRS) specific for this amino acid. Mg^{2+} is needed in this reaction.

$$aaRS + aa + ATP \rightleftharpoons (aa—AMP:aaRS) + PP_i \qquad 1.$$

In the second part of the process the amino acid specific tRNA reacts with the aminoacyl adenylate (aa—AMP) bound to the enzyme so the amino acid can bind to its tRNA.

$$(aa—AMP:aaRS) + tRNA^{aa} \rightleftharpoons aa—tRNA^{aa} + AMP + aaRS \qquad 2.$$

The sum of these two reactions is:

$$aa + ATP + tRNA^{aa} \overset{aaRS}{\rightleftharpoons} aa—tRNA^{aa} + AMP + PP_i \qquad 3.$$

The high energy bond formed between the amino acid and its tRNA makes the amino acid activated so that this energy can be used in forming peptide bonds between amino acids at the ribosome.

4.1.2. Transfer RNA

The only way for the amino acid to find its right place in the growing polypeptide chain is through the recognition between the anticodon of tRNA and the codon of mRNA. Therefore the tRNA structure was at first believed to be a single polynucleotide chain with several unusual bases disrupting double-helical hairpin regions, thereby exposing free keto and amino groups. Depending upon the specific bases the free groups may form secondary bonds to mRNA, to a ribosome or to the aaRS needed to attach an amino acid to its specific tRNA molecule. The two-dimensional structure of tRNA looks like a cloverleaf[22] with three unpaired loops and one "extra arm". At the shaft of the cloverleaf is the free 3'-end (CCA) where the amino acid can be attached. Two of the loops are named TΨC- and DHU- loop (T- and D-loop), respectively, depending upon the names of the specific bases in these loops. The third loop between these is the one where the anticodon is situated which also gives the name of this loop. The "extra arm" is named variable loop.
The 3-D shape of tRNA (Fig. 5) is, however, different from a cloverleaf (Fig. 6). It is L-shaped with a coiled structure where the T- and D-loops are close together in the knee of the L-shape. This information has come from X-ray crystallography of yeast

phenylalanine tRNA[23], [24]. The importance of the different parts of the tRNA molecule in the recognition of its specific aaRS is discussed by Normanly[25] and Abelson as tRNA identity. They stress the importance of certain base pairs and that the anticodon is a recognition element for the majority of tRNAs. Identity elements can be of two kinds. Positive elements in tRNAs for direct recognition by the cognate aaRS and negative elements in tRNAs that block the recognition.
In tRNA the structure of the cluster of nucleotides formed by the interaction of the T-loop and D-loop forms a patch that arches out from the surface of the molecule. The nucleotides in this patch are different between amino acid specific tRNA molecules. There can be insertions or deletions in the D-loop which can change the configuration of this "pocket". Klug[26] suggested that this variable pocket may "form part of a recognition system for different tRNAs, perhaps for sorting into classes for synthetase discrimination". More recent results about tRNA identity (acceptor end, anticodon and variable pocket) and which parts in the tRNA structure that cause conformational changes in binding to aaRS is described by McClain[27], [28]. Several described experiments provide strong genetic and biochemical evidence that $tRNA^{Ala}$ identity depends not only on direct recognition of G3-U70, but also on the helix deformability associated with this wobble pair and other local features. Other experiments support the proposal that both sequence and structure contribute to recognition of G3-U70 in $tRNA^{Ala}$. A general conclusion from studies of the tRNA-aaRS interaction is that the specificity function can be supplied by different nucleotide combinations in similar positions in various tRNA molecules. This feature is illustrated by the importance of some or all of the first three base pairs in the acceptor end and residue 73 of many *E. coli* tRNAs. The data also indicate that identity determinants are present in one or more anticodon nucleotides of many tRNAs, and in the variable pocket of several tRNAs.
Recently it has come information about tRNAs that they have not only this well-known canonical role during protein biosynthesis but also to perform additional functions such as acting as signaling molecules in the regulation of numerous metabolic and cellular processes in both prokaryotes and eukaryotes. Aminoacylated tRNAs have also been implicated as substrates for non ribosomal peptide bond formation in the case of cell wall formation, protein labeling for degradation, modification of phospholipids in the cell membrane, and antibiotic biosynthesis[29], [30], [31]. Another function for tRNAs is that they can act as an effective scavenger of cytochrome c, consistent with a role in regulating apoptosis.

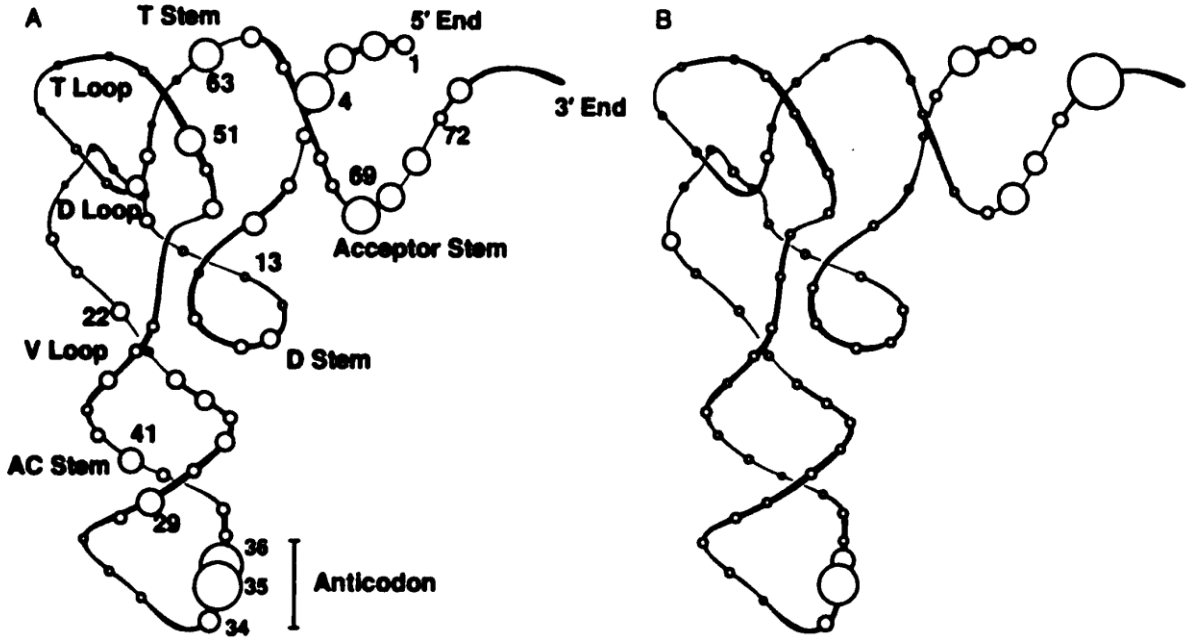


Fig. 5. Frequency data superimposed on tRNA model. Circle diameter is proportional to frequency. Predicted by structural comparisons (A). Experimentally observed tRNA determinants (B). (Adapted from Mc Clain [27, 28]).

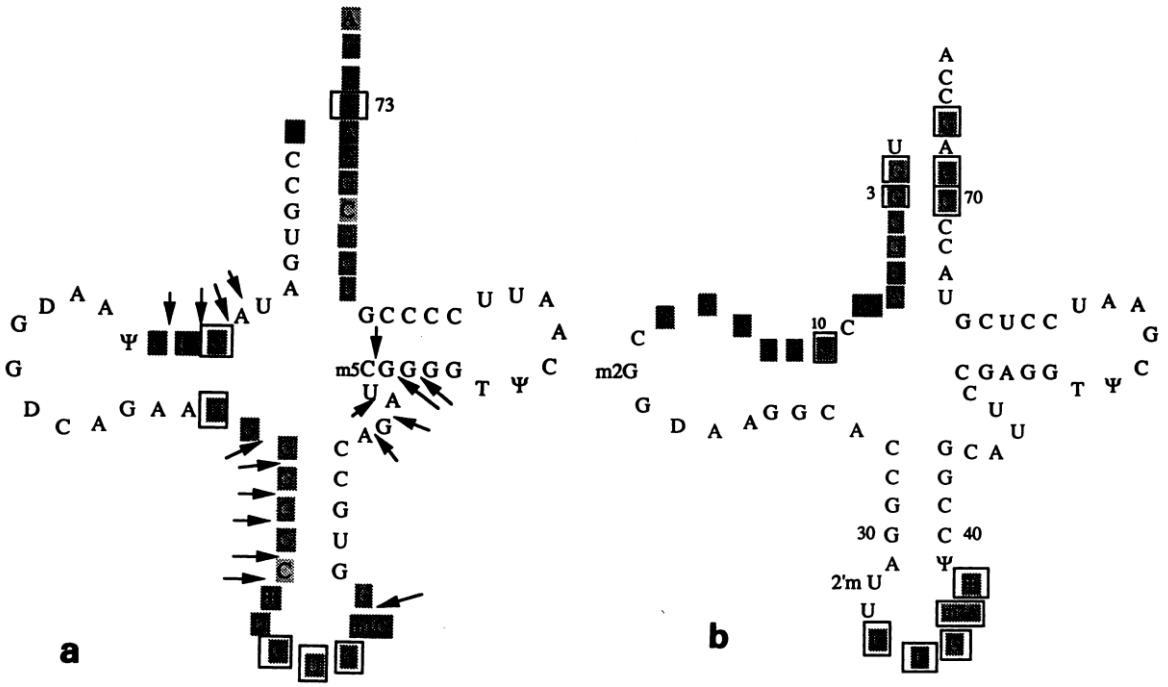


Fig. 6. Cloverleaf structures of yeast $tRNA^{Asp}$ (a) and *E. coli* $tRNA^{Gln}$ (b). (Adapted from Cavarelli and Moras [64]).

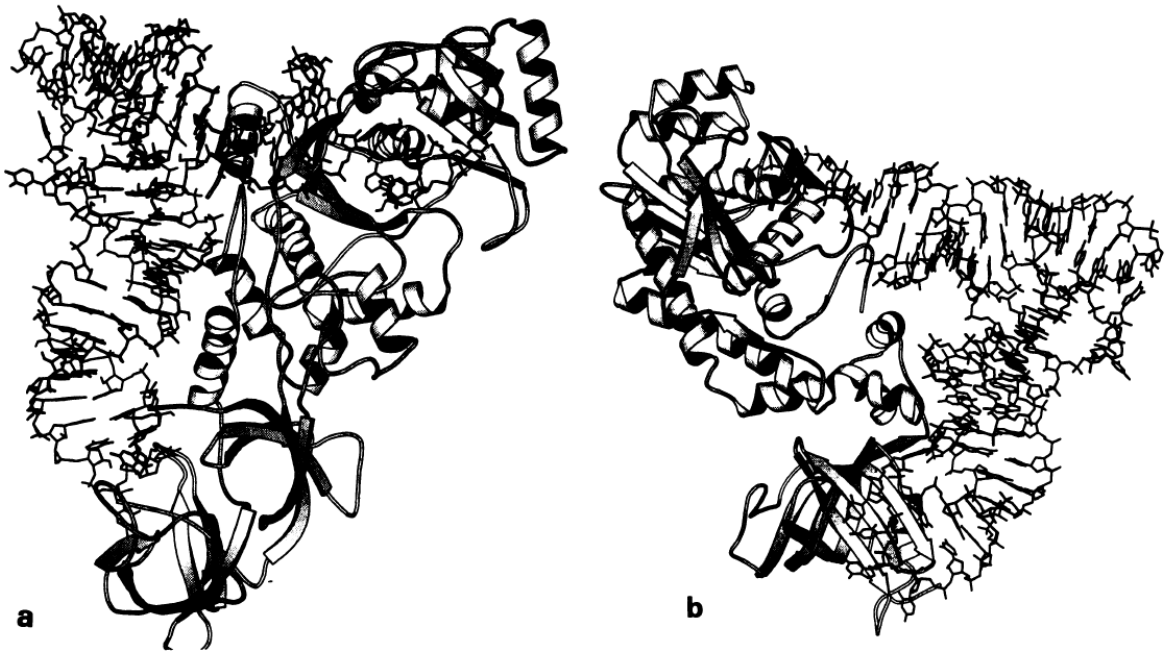


Fig. 7. Complexes $tRNA^{Gln}$ - GlnRS (a) and $tRNA^{Asp}$ - AspRS (b). The Class I GlnRS binds $tRNA^{Gln}$ from the minor groove side of the acceptor stem whereas Class II AspRS interacts with the major groove of the $tRNA^{Asp}$ acceptor stem. (Adapted from Cavarelli and Moras [64]).

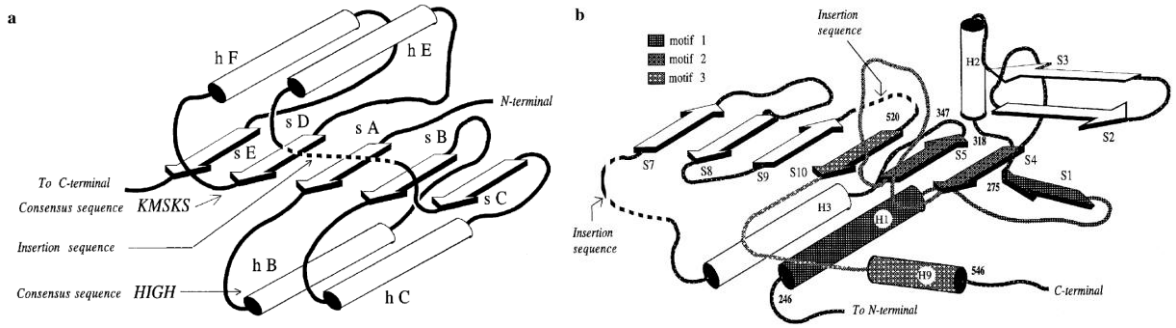


Fig. 8. Schematic drawing of the Rossmann fold, as seen in Class I aaRSs (a), and the antiparallel β sheet that forms the catalytic domain of Class II aaRSs (b). (Adapted from Delarue and Moras [70]).

4.1.3. Amino acid tRNA ligase

The importance of the correct recognition[32] between aaRS and $tRNA^{aa}$ is mentioned in discussing tRNA. In trying to understand the mechanism of amino acid activation and acyl transfer to cognate tRNAs much work has been done to find out what parts of these two 3-D structures (Fig. 7) that are in contact with each other in the aaRS-$tRNA^{aa}$ complex[33]. The aaRSs are divided into two structural classes:
Class I enzymes are predominantly specific for larger hydrophobic amino acids; they acylate the 2'-hydroxyl group of the 3'-terminal adenosine ribose of the tRNA and their amino acid activation domains incorporate parallel β-structures, and α/β folds of the Rossmann[34],[35] type (Fig. 8).
Class II enzymes are predominantly specific for smaller amino acids; they acylate the 3'-hydroxyl group of the terminal ribose of the tRNA and their amino acid activation domains are built from antiparallel β–sheets[36].
A possible origin of class-specific hydroxyl group recognition was proposed by Ruff[37] (1991) noticing that the Gln-specific aaRS from class I and the Asp-specific aaRS from class II approach their cognate tRNAs from opposite sites of the acceptor stem. Further evolutionary implications concerning the origin of the genetic code were discussed by Moras[38]. At the beginning of the progenate era there was no genetic code and only peptide-specific proto ribosome within an RNA world[39], [40], [41], [42] . One could imagine a primitive protein synthesis mechanism where a simple ribose molecule would sustain the peptide chain during elongation. A crude polypeptide synthetase reaction could constitute the transfer from the 2´OH group to the 3´OH and *vice versa*. In the second step, functions were dissociated into polypeptide synthetase and peptidyl transferase. Proteins would begin to take over the peptide synthetase. In the third step the protein synthesis machinery would grow in complexity, lending to a genetic code and the need for an interface and transfer molecules. The primordial synthetases, limited to the active site domain, would then follow parallel courses of evolution. By selecting the amino acid on one side and the transfer RNA on the other, amino acid tRNA ligases would lie at the origin of the genetic code. Lately new results for the function of aaRS to $tRNA^{aa}$ pairs has led to addition of 70 unnatural amino acids (UAAs) to the genetic codes of *E. coli*, yeast, and mammalian cells[43]. Because they are not found in the canonical 20 amino acids they provide new opportunities to generate proteins with novel properties for protein structure and function.
The enzymes vary in subunit structure and molecular weight. They are separated into four different types of structures: α, α_2, $\alpha_2\beta_2$ and α_4. Subunit organization of aaRSs is related to their separation into two classes on the basis of catalytic domain structure and short sequence relationships[44]. Class I (ArgRS, CysRS, GluRS, GlnRS, IleRS, LeuRS, MetRS, TrpRS, TyrRS and ValRS) are mostly monomeric and contain a catalytic domain with a Rossmann fold consisting of a α/β-sheet with six parallel strands and five helices[45]. Class II (AlaRS, AspRS, AsnRS, GlyRS, HisRS, LysRS, PheRS, ProRS, SerRS and ThrRS) includes α_2, $\alpha_2\beta_2$, α_4 and the catalytic domain is based on a seven-stranded antiparallel β-sheet flanked by three helices (Fig. 4). Information about 3-D structure comes from X-ray crystallography of these enzymes

specific for Gln, Met[46], Tyr[47] in class I and Asp[48] Ser[49] in class II as well as enzyme-tRNA complexes specific for Gln[50],[51], Asp[37],[52],[53] , Ser[54],[55] and Phe[56].
Some preliminary results about crystal data for LysRS from yeast[57] was obtained in our research group at the beginning of these 3-D structure determinations[58],[59],[60] . Also the MetRS from E. coli was reported only as a modified fragment after proteolytic treatment with trypsin [58]. This was crystallized at almost the same time. They also had one reference to the results from LysRS from yeast[57].
Some years afterwards the use of this monomer fragment from the MetRS dimer was reported fully active for Met and $tRNA^{Met}$ [61],[62],[63]. Also the complex between this tryptic fragment of MetRS together with the energy rich ATP was crystallized several years later[64]. More recent report for LysRS[65] and aaRS family[66],[67].

4.1.4. Amino acid tRNA ligase - tRNA recognition

There are only 20 different aaRS each specific for one amino acid. Because there are more than one tRNA specific for the same amino acid (the degeneracy of the genetic code) each aaRS must recognize more than one tRNA but still specifically aminoacylate tRNAs with different anticodons. The interaction between aaRS and its cognate tRNA occurs in two steps. The first is a diffusion-controlled bimolecular association between protein and nucleic acid. The second is a unimolecular conformational change of the initially formed aaRS-tRNA complex. In order to discriminate between cognate and noncognate tRNAs, the aaRS can use "dual discrimination" or a two-part process for recognition specificity. This means that in the first part K_m (binding step) is much higher for noncognate complexes and V_{max} (catalytic step) several 1,000-fold lower for the noncognate aminoacylation. In the second part in a homologous system noncognate complexes can have dissociation constants 100-fold or more higher than that of the cognate complex. Considerable effort has been directed at locating (on the tRNA) regions that are important for binding of aaRS[68].
Two major conclusions from this work are:
1. A bound ligase makes contact with diverse areas of the structure. The aaRS may simultaneously contact the anticodon, the 5'-side of the DHU-stem, and the acceptor terminus.
2. Most of the ligases bind along and around the inside of the L-shaped structure.
The various parts of the aaRS that are in contact with its specific tRNA substrate have been described by Cavarelli[69] and Moras and Delarue[70] and Moras. Specific identity elements in $tRNA^{Gln}$ and $tRNA^{Asp}$ in recognition of their aaRS have been found by Frugier[71]. Communication between the anticodon recognition site and active site 30 Å away is reported by Weygand-Durasevic[72]. A 17 amino acid-loop in GlnRS (residues 476 to 492) that connects two β-ribbon motifs span the anticodon binding domain and extend to the active site. The influence of a specific amino acid, Ala 294 within the motif 3 consensus of the α-subunit in PheRS from *E. coli*, especially as a determinant of the amino acid binding pocket size is investigated by Ibba[73]. Replacement of Ala 294 by either Gly or Ser, thereby increasing or decreasing the size of the binding pocket, respectively, reduces affinity for Phe. Lately the evolutionary process of aaRS is discussed by Ibba[74] and Woese[75] also for the two different classes I

and II . The special role of aaRSs for translation is discussed by Ibba[76] and Schimmel[77]. Different deseases depending on the affected aaRS involved in genetic information from mitochondrial DNA disorders as disease genes. Especially the initiation of mitochondrial protein synthesis by charging tRNAs with their cognate amino acids in the oxidative phosphorylation system is essential in the process of transferring genetic information from mitochondrial DNA[78], [79], [80], [81].

4.1.5. Protein synthesis on the ribosome

The bacterial (*E. coli*) ribosome consists of one large subunit (50S) and one small subunit (30S). The whole ribosome has a molecular weight of 2.5×10^6 (70S). In eukaryotic cells corresponding figures are 60S and 40S for the subunits, and 80S for the whole structure. Both subunits consists of rRNA as well as proteins. Protein synthesis can be separated into three parts: initiation, elongation, and termination.
Initiation starts with formation of a 30S initiation complex. This complex consists of the 30S ribosomal subunit, mRNA, formylmethionyl-tRNA (fMet-tRNA), and GTP. Three protein factors[82] specifically contributes to complex formation: IF-1, IF-2 and IF-3 .
Elongation consists of aa-tRNA binding, peptide bond formation, and translocation. Two specific proteins, Tu and Ts (or EF-Tu and EF-Ts), are involved in the process on the ribosome where aminoacyl-tRNA (aa-tRNA) binds to the ribosome[83] . Binding of aa-tRNA can be represented by 4 equations:

1. Tu·GTP + aa-tRNA ———> Tu·GTP·aa-tRNA
2. Tu·GTP·aa-tRNA·ribosomes —> Tu·GDP + P_i + ribosome bound aa-tRNA
3. Tu·GDP + Ts ———> Tu·Ts + GDP
4. Tu·Ts + GTP ———> Tu·GTP + Ts.

An older model of tRNA binding at the ribosome used only two sites: the A (aminoacyl) site and the P (peptidyl) site. Now the model seems more complicated (or complex) when these sites are handled between the ribosome's subunits in hybrid sites [19] as the tRNA moves from the A/T (or Tu mediated interaction) via A/A, A/P, P/P, P/E, and E sites. This translocation process may involve relative movement of the large and small subunits, explaining the two-subunit architecture of all ribosomes. The peptide bond formation is catalyzed by peptidyl transferase.
Termination needs two release factors, RF1 and RF2. These are proteins which can recognize the three stop (or termination) codons. RF1 recognizes UAA or UAG and RF2 recognizes UAA or UGA. The binding of RF1 and RF2 to a stop codon managed by a third release factor, RF3, and GTP [19] somehow activates peptidyl transferase so that it hydrolyzes the bond between the polypeptide and the tRNA. The specificity of peptidyl transferase is altered by the release factor so that water rather than an amino group is the acceptor of the activated peptidyl part. The 70S ribosome then dissociates into 30S and 50S subunits as the prelude to the synthesis of another protein molecule. The interaction between tRNA, rRNA and peptidyl transferase has

been discussed by Noller[84], [85] . The main interactions between tRNA and rRNA involve the extremities of tRNA: the anticodon stem/loop with 16S rRNA and the CCA terminus with 23S rRNA. One or more ribosomal proteins are involved in catalysis. They are responsible for maintaining the compact three-dimensional folding of 23S rRNA. Peptidyl transferase activity depends on this compact folding.

4.2. Methodologies used for characterization

4.2.1. Physico-chemical methods

The quaternary structures of some of the 20 different aaRSs were determined in our research group in the middle of 1970 (Paper I - IV). The structure is important for the function of these enzymes in the recognition of their tRNA-substrates. The quaternary structures of aaRSs from prokaryotes and eukaryotes are discussed by Mirande[86]. The quaternary structures of these enzymes were determined by examining the size of their possible subunits by means of molecular weight (Mr) determinations. Some of these Mr:s are determined from completed primary sequences so a comparison of these Mr:s determined with different analytical methods is now possible.
The knowledge of primary sequences of numerous aaRSs from various sources and gene technology is necessary in order to delineate the domain structures of these enzymes. Primary sequences are required in order to be able to point out specific features acquired during evolution from bacteria to mammals. In prokaryotes the complete primary sequences of 18 aaRSs were determined by gene cloning and sequencing. For those aaRSs that contain the amino acid sequences HIGH (His-Ile-Gly-His) and KMSKS (Lys-Met-Ser-Lys-Ser) the polarity NH_2-HIGH--KMSKS--COOH is conserved. As these two segments seem to be involved in the formation of the active site, this implies a common folding pattern, the carboxy-terminal moiety folding back upon the aminoterminal domain. The importance of these consensus sequences for ATP-binding is also mentioned by other authors[87], [88].
This thesis Paper I-IV concerns the structure determination of the following aaRSs :
LysRS and ValRS from *S. cerevisiae* (yeast), LysRS, AspRS, andSerRS from *E. coli*, and AsnRS, LysRS[89], SerRS, and ValRS from *B. stearothermophilus*. Paper I is a continuation of earlier preparative work[90], [91] . Experimental procedures are described in detail in Paper I or references therein. A short description will follow here.
A good yield of enzyme was important for the various structural as well as substrate recognition studies. In all preparative work each step was always controlled with respect to specific activity as well as yield. It is not possible to obtain any information of the recognition mechanism between molecules from protein preparations that are not purified enough. Therefore, the purity of the preparations was controlled to obtain optimal preparative conditions.
Different methods were used e. g. gel electrophoresis, gel chromatography, and analytical ultracentrifugation[92]. The purity of enzyme preparations was of vital importance for the studies of the molecular weights of the aaRSs. Enzymes which had not been purified enough showed a too low mean molecular mass in equilibrium

sedimentation determinations with the Yphantis[93], [94] meniscus depletion method. The reason for this was that the average Mr was determined from the slope of the line used in the calculation of the Svedberg[95] equation .
Initially, impure enzymes gave irreproducible results when the opinion was that this type of enzyme should have a molecular mass close to 100,000. However, after some time there was no doubt that different independent methods gave the same results showing real Mr values. Three previously purified ligases: LysRS [57], and ValRS [90] from yeast, and LysRS [91] from *E. coli* could now be characterized in various respects.

4.2.2. Purification of amino acid tRNA ligases from different sources

Only one example for the purification of all aaRS´s is shown here. Frozen cells of yeast (*Saccharomyces cerevisiae* C 836) were subjected to high pressure at -25ºC to break the cell walls. Extraction of this starting material with a large volume of Tris buffer (0.02 M, pH 8.0) by stirring for 30 min and centrifugation for 45 min (at 15,000xg) resulted in a crude extract.
The purification of ValRS[90] from yeast used the same steps described for LysRS[57] from yeast, with some modifications of amounts of substances, volumes and pH values.

Table 1. Purification of LysRS[57] from yeast.

Fraction	Protein mg	Total Activity units	Specific Activity units/mg	Yield %
Crude extract	38,800	5.6×10^6	140	100
Streptomycin supernatant	34,600	4.2×10^6	120	75
Ammonium sulfate	5,400	3.5×10^6	640	62
DEAE-cellulose	460	2.2×10^6	4,800	39
Amberlite IRP-64	48	1.7×10^6	35,000	30
Hydroxylapatite	11	8.7×10^5	79,000	16

The purification of LysRS [91], AspRS and SerRS from *E. coli* compared with the purification of the two enzymes from yeast described above nearly followed the same procedure. One difference was that DEAE-Sephadex was used instead of DEAE- cellulose. For more information about differences in purification steps for AspRS and SerRS, see Paper III, Table 1.
In the present investigation (Paper IV) several aaRS from the thermophilic organism *Bacillus stearothermophilus* were purified. We argued that the thermostability of proteins from this organism would favour a high recovery of native enzymes during the purification procedure and that stable crystals of the purified proteins, amenable to X-ray crystallography, would be more easily obtained.

The AsnRS, LysRS, SerRS, and ValRS from *Bacillus stearothermophilus* were all purified from the same batch of cells (Paper IV, Table I). A crude extract was first made of the cells and after an ammonium sulfate fractionation step, the enzymes were chromatographed on a column of DEAE-cellulose. Three fractiones were obtained in this step, one with LysRS, one with ValRS and one containing the AsnRS and SerRS. The asparagine and serine enzymes were separated using DEAE-Sephadex chromatography. The four aaRS were then individually purified. The overall purification ranged from 400- to 900-fold as judged by specific activity. From 1,000 g of cells approximately 10 mg of each aaRS was obtained. The purified aaRS were analyzed by polyacrylamide gel electrophoresis under denaturing conditions (SDS-PAGE) and all of the four enzymes appeared as a single, apparently homogenous band. When subjected to velocity sedimentation they formed a single peak. The LysRS could be obtained in crystalline form by dialyzing the enzyme against a solution of 1.6 to 1.8 M ammonium sulfate, containing 50 mM potassium phosphate. Another study of LysRS from B. stearothermophilus came from a group in Japan [89].

4.2.3. Preparation of tRNA

Crude yeast and E. coli tRNA was highly purified by BD-cellulose chromatography of N-phenoxyacetylated aa-tRNA[57]. The eluent contained a low concentration of $MgSO_4$ and sodium acetate buffer with a final very high concentration of NaCl and ethanol. A special notice concerning tRNAs comes from the studies of archaea where recycling of tRNAs [96] occurs with the help from aa-tRNA ligases which interact with the ribosome. One example of this is when aa-tRNAs after this type of production are selectively bound by elongation factors as EF-1 alpha in eukaryotes and archaea or EF-Tu in bacteria and delivered to the ribosome providing the growing polypeptide chain with substrates for translation elongation. The reason for this is that it is a limited substrate diffusion away from the ribosome by allowing rapid recycling of tRNAs. At the yeast ribosome the diffusion of tRNA away from the ribosome is slower than translation so some tRNA channeling takes place. When one given codon has been used to encode an amino acid during translation of a gene there is a strong tendency to encode the next occurrence of that amino acid using a codon that can reuse the tRNA that was used earlier. So tRNA molecules exiting from the ribosome remain associated with the translational machinery where they are recharged with amino acids and then readily available to be reused. Thus codon correlation is beneficial for the speed of translation.

5. THE IMPORTANCE OF THE ANALYTICAL ULTRACENTRIFUGE FOR ANALYSIS OF MACROMOLECULES

5.1. Historical overview

High speed ultracentrifugation became a reality in the 1920´s. The ultracentrifuge was initially developed by Theodor Svedberg and co-workers in these years to study gold particle size distributions (Rev. 2005[97]). For his work on the ultracentrifuge, Svedberg was awarded the Nobel Prize for chemistry in 1926. The ratio of the sedimentation velocity and centrifugal acceleration is expressed in units of Svedberg (1 S = 1 x 10^{-13} s). The name Spinco stands for the company Specialized Instruments Corporation in USA which produced the commercially available Spinco Model E analytical ultracentrifuge (Fig. 9). Today the first production of Spinco Model E is replaced by two new commercially available analytical ultracentrifuges named Optima XL-A and XL-I in 1992 (Figs. 10 and 11). These are the instruments used now which produce digital absorbance data. They are smaller and more easily maintained and serviced.

5.2. Technical aspects

Since the time with high production of analytical results from the use of Spinco Model E the interest was slowly reduced until the new Optima XL-A and XL-I could take over. Then the production of analytical results raised to the former level again and only between 2004 to 2014 with 2100 published papers on the use of analytical ultracentrifugation (Figs. 12 and 13) to assess the solution for mass, size, shape and association of macromolecules[98].

The new system is used with monochromatic light from a Xenon flash lamp and the final registration with a Photomultiplier tube for the absorbance based optical system. The Interference optical system has a Laser light source and uses Rayleigh Interference optics with a computer-controlled CCD camera detection system. Data are analyzed using specialized software on an associated computer[99].

Fig. 9. Spinco Model E Analytical Ultracentrifuge .

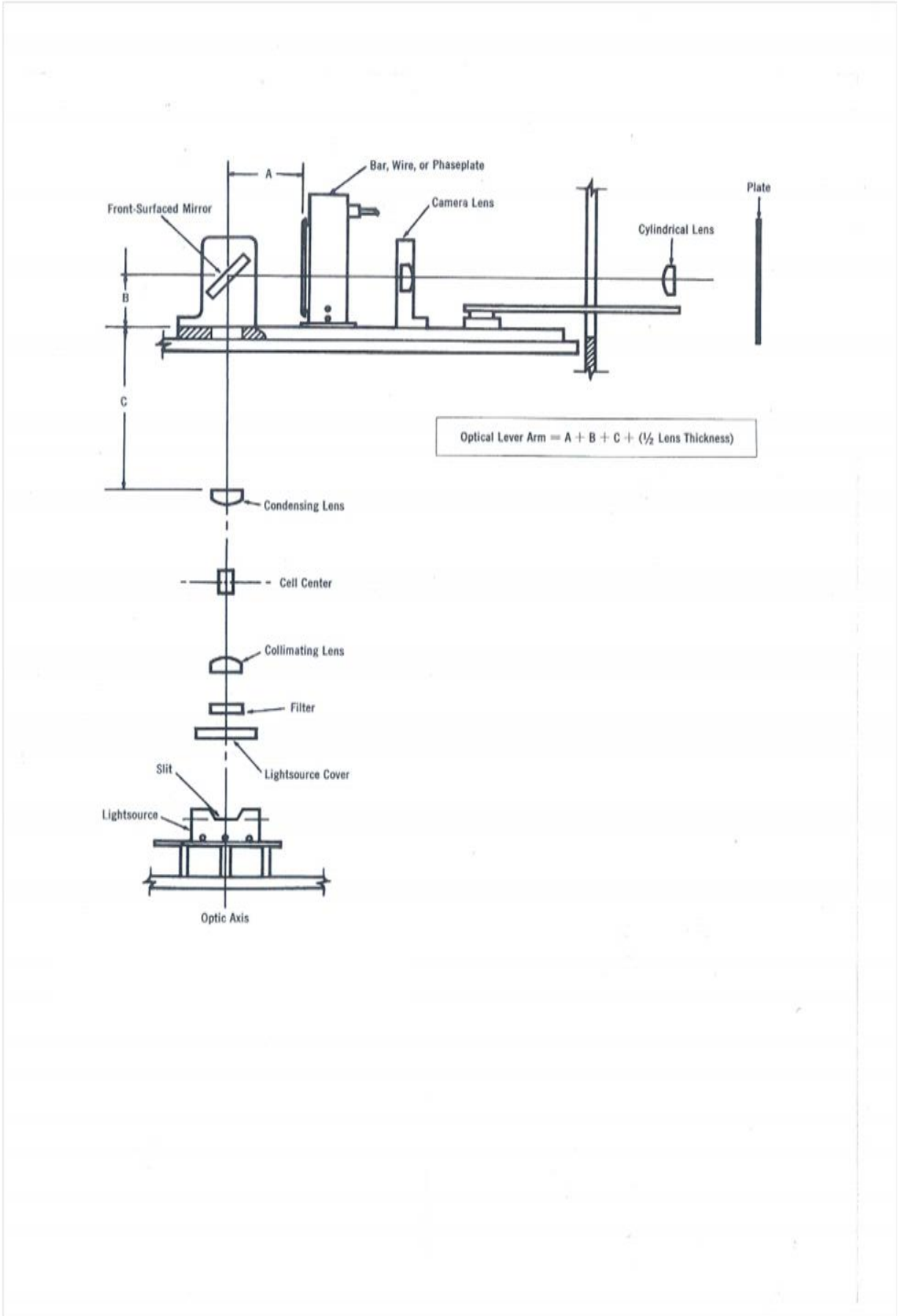


Fig. 10. Optima XL-A Analytical Ultracentrifuge

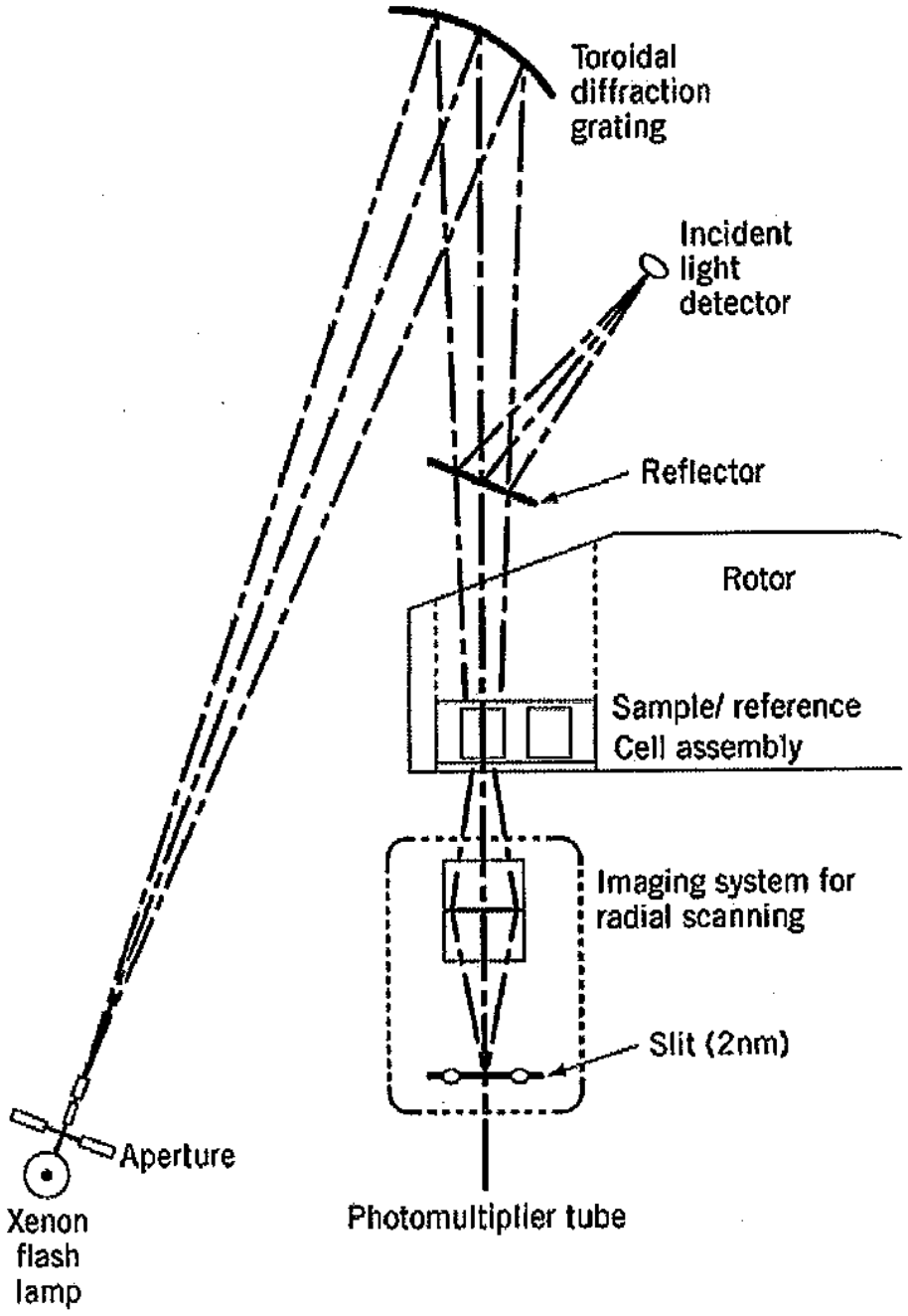


Fig. 11. Optima XL-I Analytical Ultracentrifuge

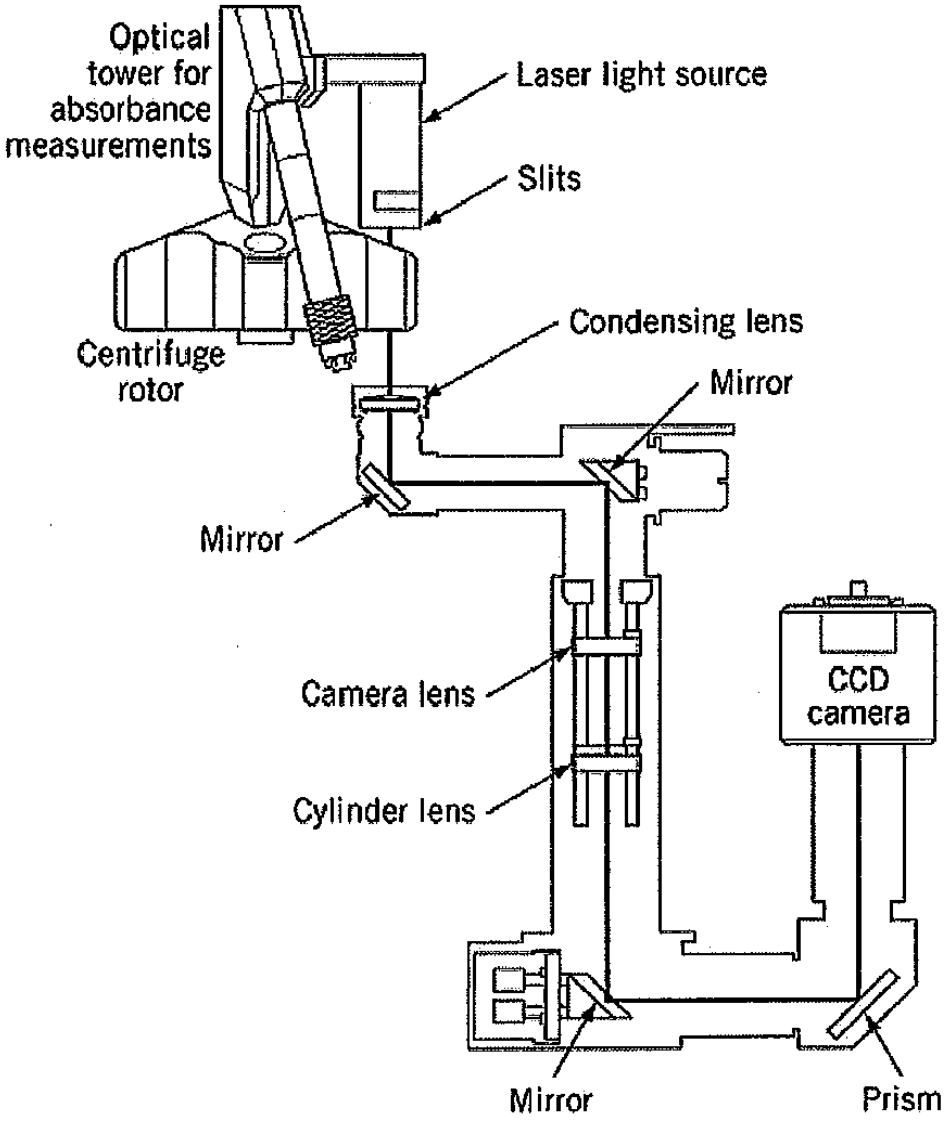


Fig. 12. Published Items in Each Year for “Analytical Ultracentrifugation”.

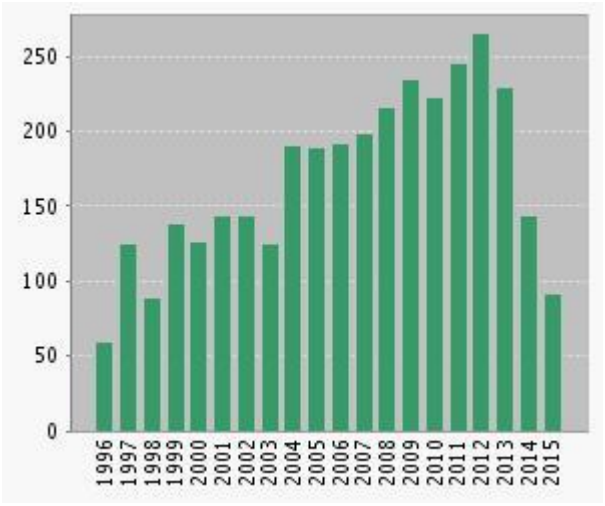
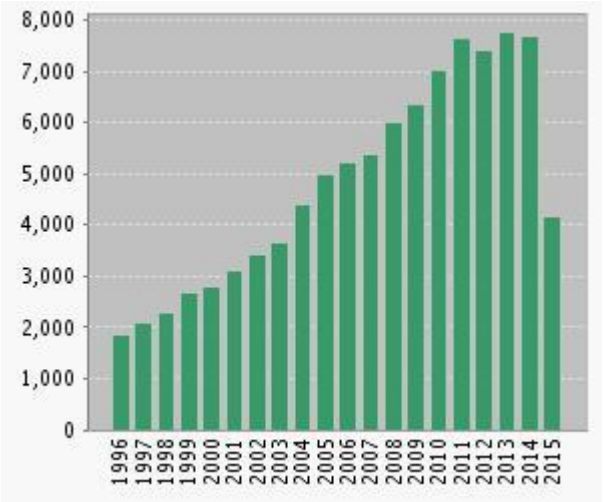


Fig. 13. Citations in Each Year for “Analytical Ultracentrifugation”.



Then the methods Sedimentation Velocity and Sedimentation Equilibrium were used as before but not manually registered because it was done digitally instead with computer directly in these new instruments[100]. The optical system was also changed from the old UV light source which could be used with the Schlieren optics (Figs. 14 and 15) or Rayleigh Interference optics (Figs. 16 and 17) with the change of special constructed parts between the light source and the lenses before the final registration was done on the film.

Fig. 14. Sedimentation concentration gradient results in Schlieren peak.

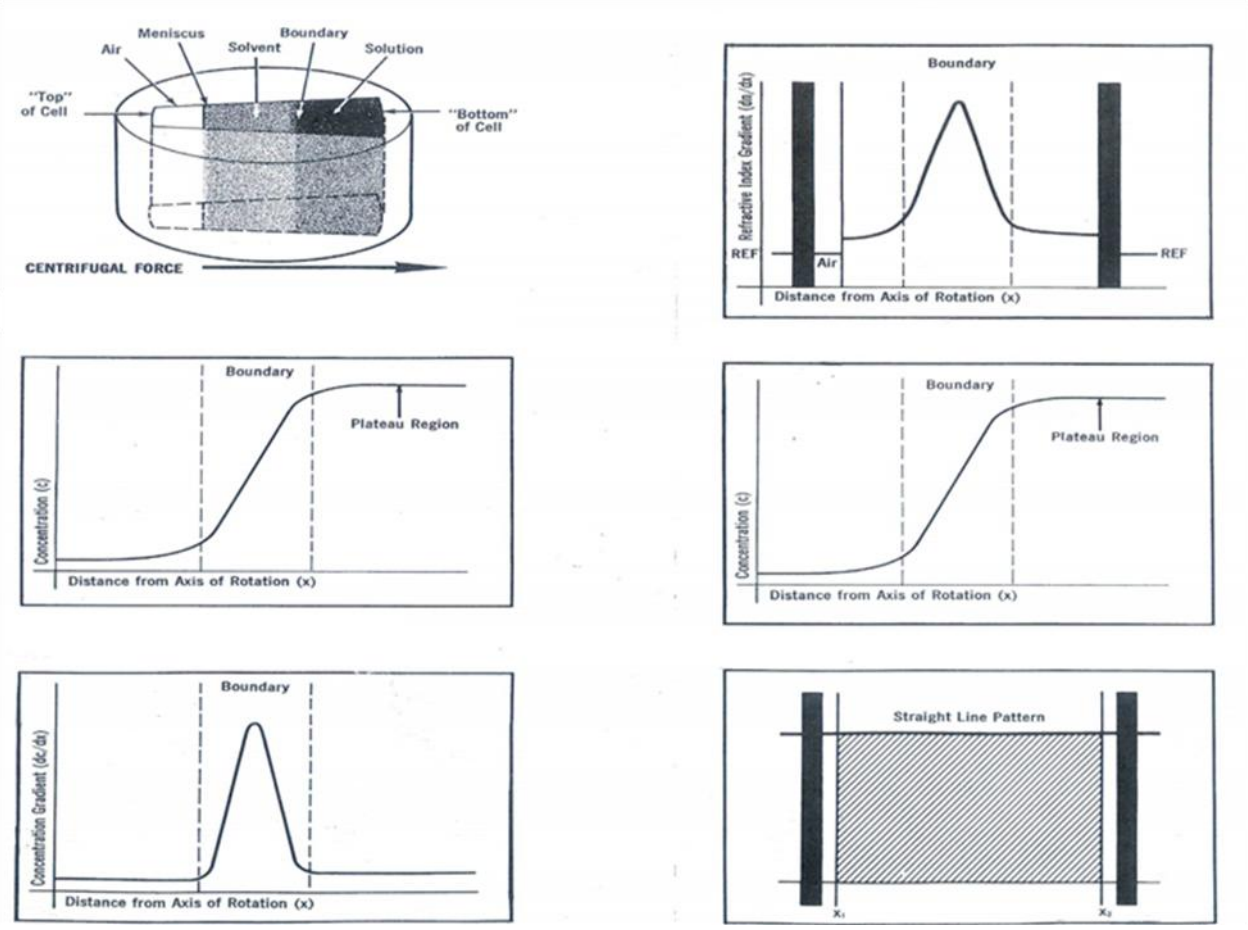
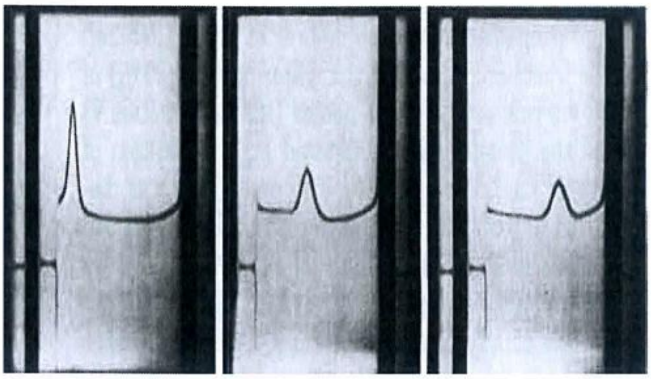


Fig. 15. Photographs (3 out of 6 possible) with Schlieren Optics of Sedimentation Velocity on Kodak Metallographic glass plate.



Velocity Sedimentation of purified Lys tRNA ligase[91]. Schlieren pictures were taken at a bar angle of 60° after 8, 40 and 64 minutes of centrifugation at 59,780 rev./min. The protein (7.15 mg per ml) had been dialyzed against 0.1 M potassium phosphate buffer, pH 7.5.

Fig. 16. Rayleigh Interference Optics.

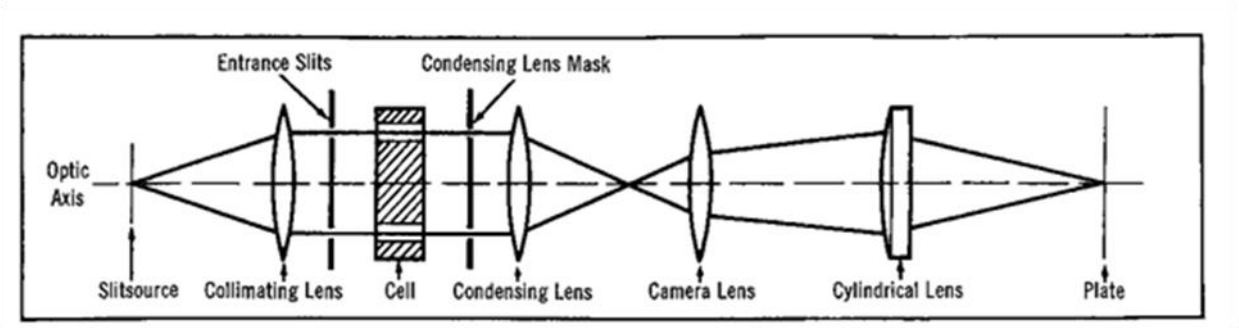
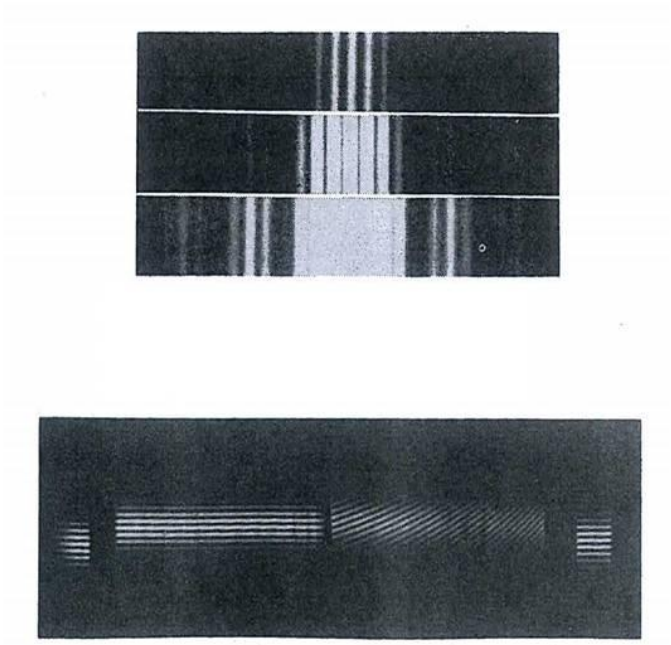


Fig. 17. Final photo with Interference Optics of Sedimentation Equilibrium on Kodak Spectroscopic II-G glass plate.



Light and Dark Bands within Diffraction Envelope (picture above).

Typical Interference Pattern (picture below).

5.3. Present state of analytical ultracentrifugation

In this received paper[101], is given and outlined the Open AUC Project. Improvements are needed for the next generation of AUC-based research. A new base instrument is described, one that is designed from the ground up to be an analytical ultracentrifuge. This ultracentrifuge will be equipped with multiple and interchangeable optical tracks with electronics and improved detectors available for a variety of optical systems. The instrument will be complemented by a new rotor, enhanced data acquisition and analysis software, as well as collaboration software. The instrument, the modular software components, and a standardized database that will encourage and ease integration of data analysis and interpretation software is described.

5.4. Analytical methods

5.4.1. Sedimentation equilibrium with analytical ultracentrifuge

These determinations were done with the analytical ultracentrifuge. According to Yphantis [93] the experimental results gave values of σ calculated from the equation:

$\sigma = d(\ln c)/d(r^2/2)$ and c is replaced by (delta y) which is received from 5 parallel Rayleigh interference fringes where all Y(i) - Y(0) is greater than 100 μ.

where c = concentration of sedimenting material at one point (i) in the rotor cell
r = radius from centre of rotation to this point (i) of sedimenting material

This was possible only at equilibrium when the centrifugal force just balanced the frictional force or when the sedimentation and diffusion coefficients are constant. When this state was obtained at the centrifugation all ln c versus corresponding $r^2/2$ points plotted in a diagram fitted a straight line as can be seen in Paper I, Fig. 3 .

From σ the molecular mass was calculated from the modified Svedberg [95] equation :

$$M = \sigma RT/\omega^2(1-\nabla\rho)$$

where M = Mr
R = gas constant = $8.314\ 10^7$ (erg K^{-1} mol^{-1})
T = absolute temperature (K)
ω = angular velocity (rad/s) (= π n/ 30) and n = speed of rotation (rpm)
∇ = partial specific volume of the solute (ml/g)
ρ = density of solution (g/ml)

5.4.2. Sedimentation velocity with analytical ultracentrifuge

These determinations were carried out with an analytical ultracentrifuge and s-values (sedimentation coefficients) were calculated from the equation:

$d(\ln x)/dt = \omega^2 s$ and $d(\ln x)/dt = 1/x\ (dx/dt)$

where x = distance from centre of rotor axis to Schlieren peak
t = sedimentation time (from full speed)

at different concentrations and extrapolated to zero concentration to obtain s^0-values as well. After correction $s^0_{20,w}$ was finally obtained.

5.4.3. Archibald approach to equilibrium

The experimental details concerning this type of analytical ultracentrifugation is explained by Ehrenberg[102] . The ratio between sedimentation coefficient (s) and diffusion coefficient (D) is obtained and used to calculate the molecular weight (Mr) from the Svedberg equation. This experimental approach is even confirmed by Trautman[103] and Crampton.

The Svedberg [95] equation gave:

$M = RTs/D(1-\nabla\rho)$

where s = sedimentation coefficient
(all other symbols are explained above)

it was so possible to calculate Mr with $s_{(20,w)}$-values obtained from sedimentation velocity determinations.

5.4.4. Gel chromatography

By means of partition coefficients, K_{av} was calculated from the equation:

$K_{av} = (V_e - V_0)/(V_t - V_0)$

where V_e = elution volume
V_t = total volume
V_0 = void volume

and the equation by Laurent and Killander[104] :

$(-\ln K_{av})^{0.5} = (\text{constant})\ r_s + \text{constant}$

It was possible to obtain the Stokes radii r_S of the enzymes from a diagram made by reference substances (Paper I, Fig. 4).
Diffusion coefficients $D_{(20,w)}$ were calculated from the Stokes-Einstein equation:

$$D_{(20,w)} = kT/6\pi\eta r_S$$

where k = Bolzmann constant
T = absolute temperature
η = viscosity

5.4.5. Amino acid composition for partial specific volume

From automatic amino acid analysis chromatography, the amino acid composition for the aaRS enzymes was determined. From these data the different partial specific volumes (∇) were calculated from the equation:

$$\nabla = \Sigma n_i V_i M_i / \Sigma n_i M_i$$

where n_i = number of amino acids/1,000
V_i = volume of amino acid
M_i = molecular mass of amino acid

When all the n_i are available and corrected to Mr one can use Mr instead of $\Sigma n_i M_i$ in the equation.

5.6. More analytical methods

5.6.1. Binding studies

According to the Scatchard[105] equation :

$$\nu/L = K(\nu-n)$$

where ν = the average number of ligands per protein molecule
L = concentration of free ligand
K = binding constant
n = the number of binding sites per protein molecule

A plot of ν/L against ν gives a straight line in analogy with the linear equation $y = kx$, and $\nu/L = 0$ when $\nu = n$ which is the intercept on the abscissa.

The theory is based on the fact that when free ligand is dialyzed through a membrane until its concentration across the membrane is at equilibrium, a direct measurement of free ligand concentration L is possible. ν is calculated from concentration of bound ligand divided by protein concentration (= binding component). The concentration of bound ligand can be obtained from the equation:

starting concentration of ligand = final concentration of free ligand +
(final concentration of free ligand + concentration of bound ligand)

Concentrations in parentheses are found on one side of the membrane.

5.6.2. Cicular dichroism measurements

From the experiments performed in a recording spectropolarimeter values were calculated according to the equation:

$$[\Theta]\lambda = \Theta\lambda \, MRW/10\,l\,c$$

where $[\Theta]\lambda$ = mean residue ellipticity (in units of degrees cm^2per decimole)
λ = wave-length (in nm)
$\Theta\lambda$ = observed ellipticity (in degrees at λ) = $(OD_L - OD_R)\lambda$ 33
MRW = calculated mean residue molecular mass (see below)
l = pathlength of light through the cell (in cm)
c = protein (or other solute) concentration (in g/ml)

MRW was calculated from the equation: MRW = Mr/n

where Mr = molecular mass (molecular weight)
n = total number of amino acid residues

6. RESULTS AND DISCUSSION

6.1. Structural and conformational determinations of ValRS, and LysRS from yeast, and LysRS from E. coli

The native Mr of ValRS from yeast determined by equilibrium sedimentation and the value calculated from diffusion coefficient (by gel chromatography) and sedimentation coefficient (by sedimentation velocity determination) (Paper I). These Mr-values can be compared with the preliminary results obtained some years before with Mr for the native enzyme estimated by approach to equilibrium[106] and calculated from sedimentation coefficient and diffusion coefficient[90]. Another study with Archibald approach to equilibrium [90] at the same protein concentration, 3.5 mg per ml, and at a protein concentration of 6 mg per ml were lower. We concluded that the true Mr for the native enzyme was derived from equilibrium sedimentation determinations described in Paper I shown in Table 2. This value is also in good agreement with the calculated value from amino acid sequence. From gel filtration determination, Chatton[107] obtained an Mr-value in good agreement with our result for the native enzyme.

The possible subunit Mr was determined by SDS-polyacrylamide gel electrophoresis (SDS-PAGE) with gel chromatography in 6 M GuHCl with DTT, and with equilibrium sedimentation in 6 M GuHCl with DTT. The different values are 10,000 too low for the former and 10,000 too high for the latter. In comparison with Mr-values from subunit determinations as well as from the native enzyme we concluded that this enzyme is a monomer of the α–type.

Mr-determinations for LysRS from yeast in its native form studied by equilibrium sedimentation resulted in different values depending on the time between preparation and determination (Paper I). We concluded that the most freshly prepared enzyme gave the true value with no contribution from low molecular weight split material. The calculated Mr-value obtained by diffusion coefficient and sedimentation coefficient was in agreement with the true value determined with equilibrium sedimentation. This value is also in close agreement with the calculated value from amino acid sequence. The preliminary Mr-results for this enzyme with approach to equilibrium [106] were much lower .

Mr-values from subunit structure determinations obtained with different analytical methods gave the results shown in Table 2. These values may be compared with the subunit Mr-values reported by Mirande[108] and Waller, and Cirakoglu[109] and Waller, confirming our results. All these subunit values show a quaternary structure for the enzyme corresponding to a homodimer of the α_2-type.

The Mr-values for the native enzyme LysRS from *E. coli* are presented with methods and results for equilibrium sedimentation as well as for diffusion coefficient and sedimentation coefficient determination with calculated value (Paper I). The calculated value from amino acid sequence is much higher and agree with two times of the subunit value from gel chromatography in 6M GuHCl. Preliminary Mr-results [83] with Archibald approach to equilibrium[91] were close to ours.

Mr-values determined for the subunit gave the results for SDS-PAGE and with gel chromatography in 6 M GuHCl as well as the equilibrium sedimentation in 6 M GuHCl with DTT. The various Mr-values are summarized in Table 2.
The quaternary structure of this enzyme must obviously be a homodimer and we concluded from these results that it must be of the α_2-type.

Table 2. Molecular weights (Mr) for ValRS, and LysRS from yeast, and LysRS from *E. coli*.

Enzyme	Mr , native. (equilibrium sedimentation)	Mr, native. (calculated from s/D)	Mr, subunit. (SDS-PAGE)	Mr, subunit. (equilibrium sedimentation in 6M GuHCl)
ValRS (yeast)	122,000	113,000	112,000	134,000
LysRS (yeast)	138,000	138,000	72,000	74,000
LysRS (*E.coli*)	104,000	108,000	62,000	54,000

Enzyme	Mr, subunit. (gel chromato-graphy in 6M GuHCl)	Mw, calculated from amino acid sequence
ValRS (yeast)	114,000	125,770
LysRS (yeast)	70,000	135,918
LysRS (*E.coli*)	58,000	115,654

6.2. Determination of binding sites

As soon as the quaternary structures were known it was possible to make conclusions about binding sites which could give valuable information about the way these enzymes recognize their tRNA substrates. The quaternary structure examinations were supported by binding experiments which resulted in one site each for Val and ATP in ValRS from yeast but two sites each for Lys and ATP in LysRS from yeast and from *E. coli*.
Km-values for LysRS from yeast and *E. coli* with tRNA substrates were calculated (Paper I, Fig. 2). It can be seen from Paper I, Fig. 8, that LysRS from yeast and *E. coli* binds two molecules of lysine per enzyme molecule. ValRS from yeast has only one binding site for valine. The same result with regard to the number of binding sites was obtained with ATP as ligand.

6.3. Thiol group determination of enzyme-ligand complexes

The Ellman[110] reaction described as: $RS^- + DTNB \rightarrow RSTNB + {}^-TNB$, was used to examine how many thiol groups that was needed (or hidden) in different enzyme -

ligand complexes (Paper II, Fig. 1). Of the 18 thiol groups obtained under denaturing conditions (with GuHCl) only 13 was accessible in the native state of LysRS from yeast. In the presence of lysine two more thiol groups were accessible but in the presence of tRNA at least four groups were protected. ATP and the adenylate complex had no effect on the enzyme in this respect.
Of the 12 thiol groups in ValRS from yeast accessible under denaturing conditions only 10 could be obtained in the native state. ATP protected nearly four groups but valine only one. Two groups were protected by tRNA but with the adenylate complex only one was protected.
LysRS from *E. coli* had three reactive thiol groups under denaturing conditions. Therefore, the difference in accessible groups was very small. Approximately one thiol group was used in all ligand reactions.
LysRS from yeast lost most of its activity when five thiol groups had reacted with DTNB. (Paper II, Fig. 1). For the ValRS from yeast this was true when only two groups had reacted. As can be seen in Paper II not much of the enzyme activity of LysRS from *E. coli* vanished in the reaction with DTNB because of its lack of sensitivity to thiol reagents[111]. As shown in Paper II, Fig. 2, out of the three enzymes there was a drastic effect of DTNB only on ValRS from yeast. The other two, LysRS from yeast and from *E. coli* were nearly unaffected by DTNB. The possible effects by DTNB on the quaternary structures of the enzymes in the native state as well as in forming amino acid activating complexes could not be determined. The studies were made (by the control of s-values) on the two LysRS enzymes from yeast and *E. coli* which were known to consist of subunits.

6.4. Conformational changes

Would it be possible that the enzymes changed their conformational structure upon binding substrates? If there is a conformational change it would show in CD-studies of the native enzymes as compared with those with bound substrates. The systems of interaction between enzyme and substrate(s) were:

1. Enzyme with corresponding amino acid.
2. Enzyme with ATP, Mg^{2+} and amino acid.
3. Enzyme with corresponding tRNA and Mg^{2+}.

These conditions were tested with ValRS, and LysRS from yeast, and LysRS from *E. coli*. The value of λ in all these experimental determinations was 220 nm, the wavelength of one of the possible negative peaks, because there the conformational change of the enzyme would contribute most. The main reason for this is that all substrates tested had no contribution at that wavelength[112] . At the wavelength 220 nm all three enzymes showed negative Cotton effects which is expected for α-helix content and the transition is n-π^* [113] with non-conservative contribution from the peptide bond[114].

When system 2 was tested with LysRS and ValRS, both from yeast, a slight difference was observed from the curve of the enzyme alone, which was not additive from the components used. This was further tested with EDTA replacing Mg^{2+}. When system 3 was tested for LysRS from yeast and *E. coli* also a slight difference occurred from the curve of the enzyme alone, which was not additive from the components used. All determinations were carried out at room temperature (after storage of enzymes and substrates on ice), at pH 7.0.

It was very hard to draw conclusions from the above studies because no drastic changes could be seen. When the ellipticity at 220 nm was used in α-helix content calculations for all three enzymes shown in Paper II, Fig. 3, the following figures were obtained for % right-handed α-helix: 24% in LysRS from yeast, 16% in ValRS from yeast, and 25% in LysRS from *E. coli*.

The differences mentioned above were 2.6% more α-helix for LysRS from yeast, and 4.2% more α-helix for ValRS from yeast, in system 2, which means a conformational change of 11% for LysRS from yeast, and 26.6% for ValRS from yeast.

In system 3, the differences were 2.6% less α-helix for LysRS from yeast, and 2.1% less α-helix for LysRS from *E. coli*, which means a conformational change of 11.0% for LysRS from yeast, and 8.3% for LysRS from *E. coli*.

These results are comparable with those obtained by Ehrlich[115] . The enzyme PheRS from yeast used by Ehrlich contains roughly twice the α-helix content compared with ours so a small difference in α-helix content in our enzymes will therefore result in a relative large calculated conformational change. The result in our case is the same as theirs with a decrease in α-helix content of the enzyme upon binding its tRNA-substrate.

6.5. Structural determinations of AspRS and SerRS from E. coli

To continue the investigations of other amino acid specific aaRSs from *E. coli* we purified eight different aaRSs from the same batch of *E. coli* (Paper III). Two of them, AspRS and SerRS, were especially characterized in terms of their molecular parameters. SerRS was examined by Katze [116] and Konigsberg at that time and published by them, so we did not publish our results for that enzyme, but for the sake of completeness these are discussed in this thesis. The purification procedures are described in Paper III and purification data are shown in Table 1 therein.

The Mr-value for the native enzyme AspRS determined with equilibrium sedimentation and from calculation (gel filtration and sedimentation velocity) shown in Table 3. Also subunit determination with SDS-PAGE and with equilibrium sedimentation in 6 M GuHCl with DTT in there. These values may be compared with subunit later obtained by Eriani[117] in agreement to ours.

Thus, the enzyme must be a homodimer with a quaternary structure of the α_2-type.

The Mr-value for the native enzyme SerRS from equilibrium sedimentation was exactly the same obtained by gel filtration and sedimentation velocity determinations. The equilibrium sedimentation Mr-value from Katze[116] and Konigsberg was slightly lower. Subunit determination with SDS-PAGE and

equilibrium sedimentation in 6 M GuHCl with DTT gave Mr values slightly higher compared with the values received from Katze[116] and Konigsberg. A similar value was derived by Härtlein[118].
These results agree well with the opinion that the quaternary structure of the enzyme is a homodimer of α_2-type. Mr-values are summarized in Table 3.
We obtained the same broad pH optimum at pH 8.5 for AspRS and SerRS in agreement with Katze [116] and Konigsberg for SerRS. Their Km-value for the corresponding amino acid at two different pH-values is in good agreement with ours at pH 7.0 for both enzymes but their Km value for ATP seems to be 5 times too high.
The aa-AMP complex forming ability was 60% for AspRS assuming one active site per enzyme molecule, and for SerRS this ability was 55%.

Table 3. Molecular weights (Mr) for AspRS, and SerRS from *E. coli*.

Enzyme	Mr , native. (equilibrium sedimentation)	Mr, native. (calculated from s/D)	Mr, subunit. (SDS-PAGE)	Mr, subunit. (equilibrium sedimentation in 6M GuHCl)
AspRS(*E.coli)*	119,000	132,000	64,000	61,000
SerRS (*E.coli*)	99,000	99,000	54,000	53,000

Mw calculated from amino acid sequence for AspRS (*E.coli)* 131,826. The native value from equilibrium sedimentation is 10,000 lower than the real value calculated from s/D. This is because of the 60% part in Fig. 4 in Paper III has been contaminated from the minor part in the chromatogram showed in this Fig.4. The s/D value is not this sensitive from Schlieren peak determination for s in velocity sedimentation.
Mw calculated from amino acid sequence for SerRS (*E.coli*) 96,828. This native value is in good agreement with both equilibrium sedimentation and s/D calculation.

6.6. Structural determinations of AsnRS, LysRS, SerRS and ValRS from *B. stearothermophilus*

Besides the main reason to provide enzymes suitable for crystal studies, it was interesting to examine especially thermostable enzymes from *B. stearothermophilus* in a high temperature kinetic point of view.
Purification steps in Paper IV, Table I. The characterization of all enzymes but one, ValRS, already determined by Koch[119] is found in Paper IV, Table III and IV.
All the purified enzymes were characterized with respect to molecular weight (Mr) and subunit structure. The data for the ValRS is not shown in Paper IV since this enzyme had already been studied by Koch [119].

In the calculations of Mr for Paper IV, Table IV the partial specific volumes from amino acid compositions (Paper IV, Table II) were as follows: AsnRS 0.739, LysRS 0.743, SerRS 0.736, and ValRS 0.738 from Koch [119] not in there.
The Mr for native enzyme AsnRS determined by equilibrium sedimentation was also calculated from gel filtration and sedimentation velocity. The molecular weights of the subunits were determined by electrophoresis in polyacrylamide gel containing 0.1 % SDS (SDS-PAGE) (Paper IV, Fig. 4) and from equilibrium sedimentation in 6 M GuHCl with DTT (Paper IV, Table III). This enzyme has a quaternary structure of α_2-type.
The Mr for native LysRS is determined by equilibrium sedimentation and calculated from gel filtration and sedimentation velocity. The subunit Mr is received from SDS-PAGE and from equilibrium sedimentation in 6 M GuHCl with DTT. This enzyme has a quaternary structure of α_2-type.
The Mr for native enzyme SerRS is determined by equilibrium sedimentation and calculated from gel filtration and sedimentation velocity. The subunit Mr is received from SDS-PAGE and from equilibrium sedimentation in 6 M GuHCl with DTT. This enzyme has a quaternary structure of α_2-type.
Finally, All the data clearly indicate that each aaRS is composed of two subunits of equal molecular weight with the exception of the valine enzyme (ValRS) which consists of only one polypeptide chain of α-type as already reported by Koch [119].
Because we also determined the enzyme ValRS (unpublished) our values can be compared with the results from Koch[119]. The Mr for native enzyme was determined by equilibrium sedimentation and also calculated from gel filtration and sedimentation velocity. The subunit Mr was received from SDS-PAGE and from equilibrium sedimentation in 6 M GuHCl with DTT. Mr-values are showed in Table 4. This enzyme is obviously a monomer of α-type.
Koch [119] obtained for native ValRS a Mr with gel filtration which agreed with our value. The subunit value determined with SDS-gel electrophoresis and agarose gel filtration in 0.1% SDS resulted in Mr 10,000 too high reported by Mirande [86]. They also concluded this enzyme to be a monomer of α-type.
Our calculated Mr value for ValRS obtained by Sephadex G 200 filtration for D-value and s-value determinations is nearly the same as that from Koch [119] obtained by gel filtration. The subunit Mr value found after SDS-PAGE was nearly 10,000 too low, but Koch [119] instead got a value 10,000 too high compared with our value.
For two enzymes, LysRS and ValRS, a special study of temperature inhibition was made. The conclusion was that the highly thermostable enzymes showed temperature dependent inhibition best explained by a conformational change of the enzymes (Paper IV, Figs. 5 to 7). We also studied the four purified enzymes with respect to their catalytic properties at high temperatures. They were all highly thermostable so that the aminoacylation rate as a function of temperature could be studied in the temperature range 25-70 °C. In Arrhenius plots the enzymes showed a marked deviation from linearity In the temperature range of 50-70 °C. This inhibition effect was not related to tRNA melting as measured by hyperchromicity. On the other hand, the K_m values for ATP and amino acid increased sharply in this temperature range in contrast to the K_m values for the tRNA substrate which showed only the expected gradual increase with temperature.

Furthermore, the ATP pyrophosphate exchange reaction is also inhibited at elevated temperatures. The inhibition observed could be explained by a conformational change of the enzymes which lowers both the catalytic activity and the substrate affinity of the ATP and amino acid sites. Because the binding sites for ATP and amino acid were more affected by the temperature induced conformation than the binding site for tRNA, this may mean that the tRNA-binding site is more located on the outer surface of the enzyme, whereas the other binding sites are located more inside.

The LysRS from *B. stearothermophilus* was crystallized but showed too low resolution (8 Å) which did not give data from crystallographic studies as good as those for LysRS [57] from yeast. Some years ago the TyrRS from the same bacterium (*B. stearothermophilus*) was crystallized in the presence of Tyr and ATP and later even three different complexes[47] between these molecules was reported. Some years before also for AspRS [48] from yeast. At that moment some important parameters had to be known before it was possible to solve the data from X-ray crystallography. One of these is now the molecular mass (Mr) for the four enzymes from *B. stearothermophilus* (Table 4).

Table 4. Molecular weights (Mr) for AsnRS, LysRS, SerRS, and ValRS from *B. stearothermophilus.*

Enzyme	Mr , native. (equilibrium sedimentation)	Mr, native. (calculated from s/D)	Mr, subunit. (SDS-PAGE)	Mr, subunit. (equilibrium sedimentation in 6M GuHCl)
AsnRS (*B. stearot.*)	127,000	120,000	51,000	51,000
LysRS (*B. stearot.*)	112,000	109,000	58,000	58,000
SerRS (*B. stearot.*)	88,000	81,000	51,000	49,000
ValRS (*B. stearot.*)	101,000	101,000	89,000	72,000

7. SUMMARY AND CONCLUSIONS

The quaternary structures of nine amino acid tRNA ligases (aaRSs) were investigated with different analytical methods. It was of utmost importance that the enzyme preparations were extremely pure, that is, no low molecular weight (Mr) from split material contribution was allowed. Another experience was that only freshly prepared enzymes showed true molecular weights (Mr) high enough and with reproductive results.

The subunits comprising the quaternary structures were investigated in two different media: SDS and 6M GuHCl containing DTT for reduction.

Out of the nine aaRSs seven comprised two identical subunits, α_2-type homodimers, and the other two were α-type monomers. These results were confirmed by binding studies with amino acid, ATP and tRNA ligands.

Enzymes comprising two subunits had two sites each for these ligands whereas in the monomeric enzymes only one site each could be found.
Conformational changes in aaRSs when they form enzyme-substrate complexes were examined with three of the studied enzymes by means of CD-determinations of α-helix content. No drastic changes could be seen but two of the enzymes showed more α-helix upon binding amino acid and ATP and two of the enzymes showed less α-helix upon binding tRNA. Studies of LysRS from yeast with its interaction to tRNA has been studied by Österberg[120].
Conformational changes in two other aaRSs of the by temperature inactivation studied enzymes from *B. stearothermophilus* showed a more temperature-induced conformation for amino acid and ATP binding sites than for tRNA binding sites.

8. THE 3-HYDROXY-3-METHYLGLUTARYL-CoA LYASE ENZYME

8.1. Background

"The enzymes forming the HMG-CoA leading to ketone bodies occur in the mitochondria, whereas those responsible for the synthesis of the HMG-CoA that is destined for cholesterol biosynthesis are located in the cytosol. Their catalytic mechanisms, however, are identical." (Voet,D. & Voet,J.G. ,BIOCHEMISTRY,3rd ed. (2004),p. 943, John Wiley & sons, inc. USA)

HMG-CoA lyase (HMGL) activity is present in *Rhodospirillum rubrum* cells grown anaerobically in the light with leucine as the carbon source[121] (Paper V). A 1.2 kb long DNA segment from *R. rubrum* was sequenced (GenBank accession number: U41280; Ref. 1). This is the first identified gene for a 3-hydroxy-3-methylglutaryl-CoA (HMG-CoA) lyase, from a photosynthetic organism. The gene encodes a 303 amino-acid-long protein with a calculated molecular weight (Mw) of 31.1 kDa.
In humans, HMG-CoA is an intermediary product of the extrahepatic catabolism of leucine. In mammalian mitochondria, HMG-CoA lyase is involved in formation of acetyl-CoA and ketone bodies (primarily acetoacetate) from HMG-CoA[122]. Acetoacetate and its metabolites are used as metabolic "fuel" in, e.g., heart, brain and kidney tissue.
HMG-CoA is also an intermediate in cholesterol biosynthesis[123], suggesting that HMG-CoA lyase could play a role as regulator of this pathway. Interestingly, HMG-CoA is implicated in hypercholesterolaemia and heart disease in humans due to the above mentioned involvement in cholesterol metabolism[124].
HMG-CoA lyase deficiency is an inherited disease where the subjects are lacking this enzyme activity as a result of the production of a truncated protein. This deficiency results in hypoglycaemia, disability to form ketone bodies and inhibition of leucine breakdown[125,126,127,128,129].

8.2. Experimental

8.2.1. Growth of cells

Rhodospirillum rubrum (strain S1) cells were grown anaerobically in the medium[130]. The experiments are described (Paper V, Figs. 7 and 9).

8.2.2. Clone isolation

To study the enzyme activity of the HMG-CoA lyase from *R. rubrum* the plasmid pÅS6 was used. It is the vector pET3a with the hmgL gene as insert, behind the inducible T7 promoter. The pÅS6 plasmid was chemically transformed into competent bacteria cells of E. coli BL21(DE3)pLysS. The over expression of the enzyme was started by the induction with IPTG.
To check the HMGL enzyme activity the transformed bacteria which had been grown and induced in the (LB+Amp) medium were used. Also as positive control Acetyl-CoA was used.
The enzyme activity for HMGL was analysed in the spectrophotometer through the production of NADH at the wavelength of λ= 340 nm.

The A.L.F. DNA sequencing reaction was used to check the plasmid pÅS6 and the hmgL gene in the vector pET3a and also the vector itself the PCR reaction was used with the T7 promoter primer (Forward) and T7 terminator primer (Reverse). These two primers together with the hmgL gene were also checked with A.L.F. sequencing when M13 fluorescence primers Forward (FP) and Reverse (RP) were used. Also primers for the middle of hmgL gene Forward and Reverse were used.
The DNA product after the PCR amplification was used for ligation into the vector pZErO digested with EcoRV and transformed into E.coli TOP10F´. The following plasmid preparation resulted in the pZErO vector with the hmgL gene (pZErO/hmgL). This plasmid was also checked with A.L.F. equipped with Argon laser (instead of the former He-Ne laser) this time.
A new protein expression system with the name IMPACT I (New England Biolabs, Inc.) was used from now on which could also give the possibility to use Western blot to identify the protein with a special anti-serum. The new vector to be used was pCYB1 which could combine the hmgL gene with a DNA sequence named Intein to receive a fusion protein from these two parts. The Intein tag on the fusion protein could be identified with anti-Intein serum in Western blot. The Intein part could later on be cleaved off with DTT from this fusion protein.
To get this DNA construction the two parts were received as:

1. pCYB1/NdeI/SapI (vector) and 2. pZErO/hmgL/ NdeI/SapI (insert)

These two parts with vector and insert were ligated with T4 DNA ligase to receive the plasmid pCYB1/hmgL which was chemically transformed into competent bacteria cells *E. coli* BL21(DE3)pLysS. Because this use of *E. coli* bacteria was not successful it was necessary to use the new recommended strain for IMPACT I instead. We received then E. coli K12 ER2267 as competent cells to be used for the vector pCYB1 and the plasmid pZErO/hmgL. The pCYB1 is Amp^r and the bacteria with this part can be grown in the (LB+Amp) medium. The pZErO is Kan^r and therefore the bacteria with this part can be grown in the (LB+Kan) medium instead. To finally check the protein over expression after the induction with IPTG it was now possible to use Western blot with anti-Intein anti serum (from rabbit) to detect the fusion protein (HMGL+Intein with CBD) where CBD is a Chitin Binding Domain part from Intein. Later on it was possible also to use anti-CBD anti serum because of this.

8.2.3. Automated sequencing

The Automated Laser Fluorescent (A.L.F.) DNA sequencing was performed on an ALFexpress DNA sequencer (Pharmacia Biotech AB). The alignment was manually manipulated for the result (Paper V, Fig.2, and text in there). In the recent control of the database UniProtKB for this hmgL gene[131] and the amino acid sequence with 303 amino acids and the molecular mass 31,138 Da (last modified May1 1997-v1) it is 100% agreement. The entry for this sequence is P95639_RHORU.

8.2.4. RNA isolation and cDNA production

RNA was isolated[132], and agarose gel electrophoresis, Northern blotting and hybridisation followed[133]. Total RNA treated with RNAse free DNAse I and in the presence of RNAse inhibitor was used for the production of cDNA with MMLV reverse transcriptase.

8.2.5. HMG-CoA lyase from hmgL gene

The PCR method was used for amplification of a stretch from the hmgL cDNA. One of the two primers used were constructed for a product of 505 bp with the complement from a BamHI RE site. This for the purpose to later clone the full length hmgL gene. The HMG-CoA lyase activity from *R. rubrum* grown in the media containing malate, leucine, mevalonate, or malate plus leucine respectively as carbon sources[134].

8.3. Recent results

The next intension was to perform mutagenesis studies on HMG-CoA lyase in order to produce a more human like protein for the identification of HMG-CoA lyase activity modulators with potential therapeutic applications. In addition, overproduction would also be optimise to produce enough protein, both native and mutant versions, for crystallization and structure determination.
This is now already solved which is reported lately[135]. Crystal structure of the recombinant human mitochondrial HMG-CoA lyase is received at 2.1 Å resolution. It was shown to contain a bound activator cation Mg^{2+} and the dicarboxylic acid 3-hydroxyglutarate (HG). The addition of the competitive inhibitor hydroxyglutaryl-CoA (HG-CoA) was necessary for generation of uniform diffraction quality crystals. These inhibitor studies suggested that HG-CoA is not an efficient substrate for HMG-CoA lyase[136]. In addition, crystal structures of two bacterial HMG-CoA lyases have been reported[137] .
The identification and characterization of an extramitochondrial human HMG-CoA lyase[138] was also reported lately. From plasmids constructed for this it was first a trial to use over expression of the protein in *E. coli*. This was not successful because of the production of insoluble material so instead another bacterium *Pichia pastoris* was used with the final positive result.

9. THE HD PROMOTER INITIATION DOMAIN SEQUENCE

9.1. Background

I was given the plasmid pAop2CS (C39S) [139] for transformation into the bacterium *E. coli* BL21(DE3)pLysS. The origin of this was pAR3038[140],[141] . The concentration of the polypeptide AntpHD was calculated from the known amino acid sequence with 68 amino acids with the contributions especially from the three Trp, Tyr, and Phe which have absorption at 280 nm wavelength measured in the spectrophotometer.
The calculated molecular weight (Mw) for this AntpHD was used as 8.629 kDa with an isoelectric point (pI) of 10. Which means that the polypeptide will be positive of charge in a buffer with pH 7.5 which was used in our experiments. So the column with Bio-Rad 70 resin which is for separation of positive ions is useful.

9.2. Methods and Results

The first thing to do after the transformation of the bacterium *E. coli* BL21(DE3)pLysS with the DNA material in the received plasmid pAop2CS (C39S) was to check the sequence of the Antp gene with the mutation named Antennapedia. This was done with the A.L.F. DNA Sequencer from Pharmacia, Uppsala Sweden with He-Ne laser (633 nm) which emits red light[142] or with Argon laser (488 nm) which emits blue-green light from Amersham Pharmacia Biotech Sweden which also was used in this time. The DNA sequence of this Antp gene was already known[143] and could easily be checked and compared of this reason. The result was correct from these examinations so it looked safe to use this transformed bacterium for preparation of the AntpHD with over expression by means of IPTG of the T7 system[139]. The opinion was that this originally received gift from the research group in Basel, Switzerland was exactly the same which had been used with the successful results even from NMR determinations [143] in the research group in Zürich.
From this time and several years to come it was a very hard work to make all possible steps in the purification procedure extremely well performed also with many changes to receive even better results if possible? Nothing worked to get the received product checked finally with NMR. It was not until late in the year 2009 it was decided to send the DNA material from plasmid pAop2CS which was the gene Antp in the vector pET3c for examination to Eurofins MWG BIOTECH in Germany. The answer then was unexpected that no DNA material could be found and analysed!
The reason was that the company used a T7 promoter primer with 3 C bases in the 3´ end and it should be 3 G bases as a complement for these in the T7 promoter in the pET3c vector. It could be seen now that one of these 3 G from the DNA bases in the plasmid vector sequence was missing! An unexpected information also for the analysing company Eurofins MWG in Germany. They also checked this result extra with one T7 promoter primer where only one C base out of the three in the 3´end was eliminated. Now this one could be fixed and the DNA sequence was received as expected! This result is in agreement with other examinations of T7 promoters[144],[145],[146],[147] with bases checked for importance to T7 RNA polymerase transcription of the gene. It says that the T7 promoter with 22 bases beginning with 5´ TAA TAC and the following GAC TCA continues with CTA TAG GGA. Here the first G in the GGG sequence is numbered +1 so the final A is numbered +4. Moreover the bases from -4 to +5 which are the TATA and GGG AG is the initiation domain whereas the former bases from -12 to -5 which are the CGA CTC AC is the binding domain.

It is only two G bases where it should be three G bases in the T7 promoter sequence from +1 to +3. All other bases in the T7 promoter are correct however. Because the three G bases signed +1 to +3 are from the middle of the DNA sequence for initiation of transcription[148, 149] for the much later following bases in the Antp gene it is critical when one of these G bases is missing as it is here! Therefore no mRNA is produced and the translation from this actual gene to AntpHD will never occur. The importance of this part with intact three G bases was also noticed from DNA sequencing with the company MWG eurofins when their T7 promoter primer could only be fixed when they had to use one C base less in the complementary DNA sequence in the T7 promoter primer. However the DNA bases in the Antp gene were checked to be correct with ALF DNA sequencing originally.
It was necessary to make a new DNA construction of the plasmid with the same original gene Antp with a correct vector with intact T7 promoter to use for over expression of the AntpHD. Now with the new vector pET21b for this instead. The new DNA construction which should be taken from the original plasmid with Antp gene in the pET3c vector which first must be cut out from this and after this ligated into the new pET21b vector. Now the problem was to find the correct restriction enzymes (REs) for this in these two DNA materials. It was necessary to use PCR with specially constructed primers to make correct RE sites for both.

FP Antp (27 bp): 5´ GGG CATATG CGC AAA CGC GGA AGG CAG 3´ (for NdeI)
RP Antp(27 bp): 5´ GGG GAATTC TTA ACC CGG CTC GCC CTT 3´ (for EcoRI)

The two resulting REs were EcoRI and NdeI finally and after the fragments from the materials which were used were cut off and eliminated the pET21b was now changed to pET21a with 5 Pro instead of 6 His because of the change of reading frame in the received DNA sequence. Then the procedure with a Ni column for production of AntpHD could not be used instead of the column with Bio-Rad 70 resin. The Work with this procedure which were used before [139] is still going on to receive material for AntpHD examinations pure enough and with the correct concentration for a new determination with mass spectrometry or even NMR. Later on also for binding experiments.

9.3. Concluding remarks

The homeobox genes which regulates nuclear proteins that acts as transcription factors during normal development are DNA sequences originally identified in *Drosophila* [3]. These proteins have different HD and are recognizing and binding sequence-specific DNA motifs. The specificity of this binding has the effect to activate or repress the expression of downstream effector target genes [2]. The class I HD (Hox genes) is mentioned in embryonic development and in the control of cell proliferation which can result in pediatric cancers as abnormal development of human congenital, somatic, and metabolic defects involving mutations in homeobox genes. The mechanism for Hox proteins in binding to DNA in multiprotein complex[150] is also studied. Early mammalian embryonic development with the stem cell transcription factor Nanog[151], [152] is also reported.
To continue the comment in introduction about different fields for HD effects it can be noticed that the studies with mice have given valuable information concerning “mechanisms regulating insulin secretion and metabolism in related diseases such as obesity and type 2 diabetes”[153]. Here the LIM-HD transcription factor isl-1 in the pancreatic endocrine cells is involved in both islet development and insulin secretion together with leptin in some way.

It has also come new information concerning the participation of HD as AntpHD for $CD8^+$ Cytotoxic T Lymphocyte cells (CTL)[154] for treatment of patients with cancer. It was the effect of transport into liposomes into the endoplasmic reticulum with the soluble AntpHD recombinant peptide which can induce T cell responses and this effect can be used as vaccine in this way. It has also been studies of the DNA binding human HD oncoprotein HOX11 as transcription factor for spleen development in embryogenesis. It is also associated with T cell acute lymphoblastic leukemia[155] in children. So HOX11 has a functional role in both normal development and malignancy. A special nuclear oncoprotein TLX1/HOX11 is studied with leukemic T cells[156]. Another homeobox protein MSX2 is studied for malignant melanoma[157]. Since the early studies in evolution reported from Charles Darwin (Darwin, Ch. 1859) the studies of eye evolution[158],[159] is reported lately. Another contribution to the description of eye development and also disease comes from studies with the homeobox gene Otx2 [160] .

10. ACKNOWLEDGEMENTS

I wish to express my thanks to all those who have support me during the years of this work. In this respect I should like to especially mention:

Prof. Lars Rymo my first supervisor at the Medical Biochemistry department for his valuable advise and never failing interest in my research work.

Prof. Åke Strid and Prof. Martin Billeter during my time in their respective research groups at the department of Biochemistry and Biophysics. They have given most valuable support and professional criticism to me concerning the performance of analytical methods in my research work.

Prof. Hans Elwing for his work as my new supervisor in correcting my thesis with valuable critical comments.

Prof. Anders Blomberg for his work as my Examinator also after valuable discussions during long time before.

Prof. Jan Rydström for his work as my supervisor and Examinator for my thesis before concerning my Licentiate Degree of Philosophy.

Dr. Daniel Farkas and Dr. Jonas Fredriksson my dear colleagues who have given valuable advise and support to me through all the years of analytical work we have done in our research groups.

Brian George from Beckman Instruments, Inc. Spinco Division Palo Alto CA USA for his excellent work to find the news magazine Beckman Fractions 1966 #2 for this company where I used the reference for the invaluable computer program (PAS001C) written in FORTRAN II 1965 by P. A. Small & R. A. Resnik concerning Yphantis Meniscus depletion method for Sedimentation Equilibrium runs with the Analytical Ultracentrifuge Spinco Model E from Beckman Instruments, Inc.

Lars Nordvall and Bruno Källebring for all their help with my computer questions as well as other more technical problems at the Lundberg laboratory.

Andreas Chadien and Ulf Johansson who have been most helpful with necessary work for daily routines of all kinds at the Lundberg laboratory.

All my friends through the years at the Göteborg University not mentioned here but always remembered with gratitude.

Finally Johan Lundvik, my son, for his never failing interest in my research. Without his great support in this respect this thesis would never have been finished. Thank you so very much for this.

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