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A novel medium for expression of proteins selectively labeled with ^{15}N-amino acids in *Spodoptera frugiperda* (Sf9) insect cells

Michael Brüggert, Till Rehm, Sreejesh Shanker, Julia Georgescu & Tad A. Holak*
Max Planck Institute for Biochemistry, D-82152 Martinsried, Germany

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Abstract

Whereas bacterial expression systems are widely used for production of uniformly or selectively ^{15}N-labeled proteins the usage of the baculovirus expression system for labeling is limited to very few examples in the literature. Here we present the complete formulations of the two insect media, IML406 and 455, for the high-yield production of selectively ^{15}N-labeled proteins in insect cells. The quantities of ^{15}N-amino acids utilized in the production of labeled GST were similar in the case of bacterial and viral expression. For the most studied amino acids essential for insect cells the ^{15}N-HSQC spectra, recorded with GST labeled in insect cells, showed no cross labeling and provided therefore spectra of better quality compared to NMR spectra of GST expressed in *E. coli*. Also in the case of amino acids not essential for Sf9 cells we were able to label a defined number of amino acid species. Therefore the selective labeling using the baculovirus expression vector system represents a complement or even an alternative to the bacterial expression system. Based on these findings we can provide a first simple overview of the network of the amino acid metabolism in *E. coli* and insect cells focused on nitrogen. For some amino acids the expression of labeled proteins in insect cells can replace the cell-free protein expression.

Introduction

The baculovirus based expression systems are one of the most powerful expression systems known in the biochemistry nowadays. One of the major advantages over bacterial expression systems is represented by the ability to allow easy production of functional heterologous proteins like, enzymes (Lawrie et al., 1995; Kumar et al., 2001), antibodies (Brocks et al., 1997), or receptors (Cascio et al., 1995; Zhu et al., 2001). Moreover, the cell machinery present in insect cells is the garantor for proper folding and posttranslational modifications, essential for the biological functionality of a large number of foreign proteins.

The most important parameters that influence protein yields in the baculovirus system are: cell density at the time of infection (Jesnionowski et al., 1997), the multiplicity of infection (MOI) (Jesnionowski et al., 1997; Bedard et al., 1994), the age and the composition of the medium (Doverskog et al., 2000; Jesnionowski et al., 1997; Chiou et al., 2000). To obtain high cell densities, and therefore high protein yields, several media (Doverskog et al., 1998; Ferrance et al., 1993) and feeding strategies (Kim et al., 2000; Doverskog et al., 2000; Mendonça et al., 1999; Chiou et al., 2000) have been developed to enhance cell growth. The identification of essential and non-essential amino acids for cell growth was one of the most important factors. So far, alanine, cysteine, glutamic acid, glutamine, aspartic acid and asparagine were found to be non-essential amino acids (Öhman et al., 1996; Doverskog et al., 1998; Mitsuhashi, 1982). Further agents influencing cell growth are growth factors, vitamins and other compounds (Öhman et al., 1995; Mendonça et al., 1999) which are provided by chemically not defined substances, like yeastolate or fetal calf serum (Drews et al., 1995; Ferrance et al., 1993). In general the serum can be replaced by lipid mixtures but the yeastolate contains additional nutrients which, al-

*To whom addressed should be addressed. E-mail: holak.biochem@mpg.de

though not completely identified, are beneficial for growth. The use of media without yeastolate leads to a dramatic decrease of growth rate and maximal cell density (Kim et al., 1999). Serum and yeastolate are also sources of free amino acids (Mitsuhashi, 1982) at different extent.

NMR-based structural studies and NMR-based ligand binding studies require at least selectively ^{15}N-labeled proteins. On the one hand for the expression of uniformly labeled proteins in *E. coli* ^{15}N-ammonium chloride is used as a sole source for nitrogen, whereas commercially available media for uniform labeling in insect cells contain all ^{15}N-amino acids, which increase the costs dramatically. On the other hand for selective labeling in bacteria a medium is applied containing all amino acids similar as for insect cells and comparable costs can be expected. Unfortunately, only few reports on labeling proteins in insect cells can be found in the literature (Creemers et al., 1999; DeLange et al., 1998), in addition the total composition of the used media has been kept secret.

This work describes the novel medium IML406 for selective labeling proteins expressed in Sf9 insect cells. This medium was compared with other widely used insect media using growth and expression studies. The content of amino acids in IML406 was optimized for selective ^{15}N-labeling with respect to the free amino acid supplied by serum and yeastolate. For the purpose of labeling one of the criteria required from the studied model protein should be primary a good yield of expression in *E. coli* and Sf9 insect cells. It also should possess a high stability and its size must be in the typical range for many proteins expressed with the baculovirus expression vector system. The model protein of choice for selective ^{15}N-labeling in Sf9 was glutathione-S-transferase protein (GST) from *S. japonicum*, which is widely used as purification tag. The first step was to focus on amino acids used for direct labeling in *E. coli* and glycine, leucine, lysine, phenylalanine and valine were chosen. In a second step, the medium IML455, a derivative from IML406, was utilized to perform ^{15}N-labeling studies with aspartic acid, glutamic acid and ammonium chloride in Sf9 in order to investigate pathways described by Drews et al. (2000). These amino acids are known to take part extensively in transamination reactions in *E. coli* (Waugh, 1996). All results of selectively ^{15}N-labeled GST were compared with the results of the bacterially expressed protein. We also discuss the degrees of ^{15}N-incorporation for several essential amino acids based on the results of quantitative amino acid analyses of yeastolate and serum and propose solutions for enhancing the incorporation of ^{15}N-amino acids to values similar to those found in in-vitro translation systems.

Materials and methods

Media formulations

One liter of insect cell medium IML406 contained 10 ml of 100 × MEM-vitamins (Gibco), 10 ml of 100 × lipid mixture (Sigma) and 20 ml of 50 × yeastolate-ultrafiltrate. The mass of inorganic salts per liter was: KCl, 2.87 g; $CaCl_2 \times 2H_2O$, 1.4 g; $MgCl_2 \times 6H_2O$, 2.2 g; $Mg_2SO_4 \times 7H_2O$, 2.8 g; $NaHCO_3$, 0.35 g; NaH_2PO_4: 0.9 g. The amount of trace elements was: $CoCl_2 \times 2H_2O$, 0.07 mg l^{-1}; $FeSO_4 \times 7H_2O$, 0.55 mg l^{-1}; $MnCl_2 \times 4H_2O$, 0.03 mg l^{-1}; $(NH_4)_2Mo_6O_{24} \times 4H_2O$, 0.06 mg l^{-1}; $ZnCl_2$, 0.06 mg l^{-1}; vitamin B12, 0.24 mg l^{-1}. The amount of trace elements, inorganic salts and vitamins was not changed in later modified media of IML406. Amino acids were added to 1 l of IML406 in following amounts: L-glutamine: 1 g; L-arginine, 0.7 g; L-asparagine, 0.35 g; L-aspartic acid, 0.35 g; L-glutamic acid, 0.6 g; glycine-HCl, 1 g; L-histidine, 0.2 g; L-isoleucine, 0.175 g; L-leucine, 0.4 g; L-lysine-HCl, 0.625 g; L-methionine, 0.08 g; L-phenylalanine, 0.25 g; L-proline, 0.35 g; L-serine, 0.55 g; L-threonine, 0.175 g; L-tryptophan, 0.1 g; L-tyrosine, 0.1 g; L-valine, 0.2 g; L-cystine, 0.08 g. The medium IML455 contained 0.265 g l^{-1} NH_4Cl instead of L-aspartic acid and L-glutamine. IML406 and IML455 media were both supplemented with 5 g l^{-1} glucose and the osmolality was adjusted to 310–330 mOsm kg^{-1} with solid NaCl, pH was set to 6.2–6.3 and adjusted with 5 M NaOH. The media were sterile filtered through 0.22 μm filter unit (Millipore). For expression of selectively ^{15}N-labeled GST the desired amino acid was used in its ^{15}N-version. The medium KBM10 was prepared as described by Doverskog et al. (1998), whereas the commercial medium SF900II (Life Technologies) was used with and without 5% (v/v) fetal calf serum (Sigma). IPL41 was prepared following the formulation provided by Life Technologies and was supplemented with 4 g l^{-1} yeastolate-ultrafiltrate (Life Technologies). All used insect media were completed with 30 mg l^{-1} Gentamicin (Gibco) and 0.025 mg l^{-1} Fungizone (Gibco).

The formulation of bacterial media for selective labeling of proteins was prepared as described by Senn

et al. (1987). For uniformly labeled ^{15}N-GST expressed in *E. coli* a medium described by Riesenberg et al. (1990) was used.

Maintenance and adaptation of Sf9 insect cells

Spodoptera frugiperda (Sf9) cells were maintained in spinner flasks as suspension culture or in 75 cm^2 tissue culture flasks as adhesion culture. The cells cultured in adhesion cultures were passaged at 80–90% confluency and finally diluted to 30% confluency. The suspension cultures were routinely passaged at cell densities of 1.8–2.0 $\times$ 10^6 cells per ml and diluted to a final cell density of 0.3–0.4 $\times$ 10^6 cells per ml. All cultures were incubated at 27 °C and the suspension cultures were stirred at 95 rpm.

The cells had to be stepwise adapted to new media conditions. Therefore after seeding at 30% confluency in a 25 cm^2 tissue culture flask they were exposed to a medium containing 50% of the new and 50% of the initial medium. At confluency the Sf9 cells were diluted in the same mixture. After reaching a constant growth rate the cells were exposed to a 75% new medium and passaged until a constant growth rate was reached again. In the final step 100% of the new medium was used. After adaptation to the new conditions the cells were maintained in suspension culture for further growth and expression studies.

Plasmids and viral stocks

For the viral expression of GST in insect cells, the plasmid pAcG2T was chosen. The plasmid was used for co-transfection with linearized BaculoGold virus DNA (Pharmingen). For purification and determination of the titer of the recombinant baculovirus, a plaque assay was performed as described elsewhere (O'Reilly et al., 1992). For bacterial expression of GST the plasmid pGex4T2 (Pharmacia) was transformed in *E. coli* BL21(DE3).

Growth and expression studies

For growth studies adapted Sf9 cells were maintained for 10 passages in adhesion culture in the desired medium. After this period the insect cells were transferred to suspension culture and grown in 50 ml of the medium at an initial density of 3 $\times$ 10^5 cells per milliliter. The density and vitality of the cells was measured after 24, 48, 72, 96, 120 and 144 h of culturing. The test expressions were performed in 100 ml suspension cultures. The cells were infected with a MOI of 8 at a cell density of 2.0–2.2 $\times$ 10^6 cells per milliliter. After 60 hours the cells were harvested by centrifugation at 900 g and 4 °C. The GST was purified (vide infra) and the quantity was determined using method of Bradford.

To investigate the influence of the amount of a certain amino acid provided by yeastolate or serum on cell growth and protein yield, this amino acid was not added separately to the medium. The cells were grown in 25 cm^2 tissue culture flasks using the modified versions of medium IML406. After ten passages in adhesion culture the insect cells were transferred into the suspension culture for performing growth and expression studies. The cell density at the time of infection was 1.5$\times$10^6 cells per ml. Cells were harvested after 50 h.

Large-scale expression of the GST

For expression of the protein in insect cells a 2 l suspension culture in the desired medium was infected with the virus at a MOI of 8 at a cell density of 2.0–2.2 $\times$ 10^6 cells per ml. After 60 h the cells were harvested by centrifugation at 900 g and 4 °C. The pellets were frozen at −80 °C. For bacterial expression of the GST protein 25 ml of the bacterial growth medium was inoculated with one colony. The culture was incubated for 14 h at 37 °C under shaking (220 rpm). The entire preculture was used to inoculate 1 l medium under the same fermentation conditions. At an optical density of 0.7 at 600 nm expression was induced with 1 mM IPTG final concentration. After 4 h the cells were centrifuged at 5000 *g* and 4 °C and stored at −80 °C until use.

Cell pellets were resuspended in buffer A (50 mM Na_2HPO_4 pH 7.4; 250 mM NaCl; 5 mM DTT) and lysed by sonification at 60 cycles for 2 min. After centrifugation, the supernatant was incubated for four hours with glutathione sepharose FF (Pharmacia). The resin was washed with ten column volumes of buffer A followed by the elution of GST with buffer A including 50 mM reduced glutathione. The protein was dialyzed in a Spectropor 8 MWCO dialysis tube against buffer B (50 mM Na_2HPO_4 pH 7.0; 250 mM NaCl; 5 mM DTT) and concentrated to 1–2 mg l^{-1} using a Centriprep10 (Amicon). The total amount of GST was determined by Bradford assay.

Final samples for NMR analyses have been prepared in Buffer B containing 10% D_2O and concentrated with a Centricon 10 (Amincon). The final protein concentration ranged from 0.2 to 0.8 mM.

Analytical methods

Cell counting and determination of the mean particle size were performed with a particle size analyzer Coulter Z2 (Beckmann Coulter). The viability of cells was determined by trypan blue exclusion. The osmolality was determined using a freezing point osmometer Osmomat 010 (Gonotec).

The quantitative analyses of free amino acids were performed with an amino acid analyzer LC3000 (Eppendorf). The serum was deproteinated by addition of 200 µl 10% sulfosalycylic acid to 800 µl serum. Before loading of samples yeastolate was diluted 1:80 and 1:160, the serum 1:1 and 1:2. The amino acids were separated by a strong cation exchange column PEEK and detected after post-column derivatisation with ninhydrin. The molecular mass of GST was determined by MALDI-TOFF mass spectrometry.

NMR spectroscopy

All NMR spectra were acquired at 300 K on a Bruker DRX-600 spectrometer. HSQC spectra (Mori et al., 1995) were recorded with 128 increments in the indirect ^{15}N dimension with a number of scans varying from 128 to 1024 depending on individual samples. For bacterial expression 128 scans were used for ^{15}N-glycine, ^{15}N-glutamic acid, ^{15}N-tyrosine and ^{15}N-valine labeled GST. For the other amino acids we applied 320–400 scans. For expression in insect cells 380–512 scans for recording the HSQC spectra were used. The spectrum of GST labeled with 5 mM ^{15}NH4Cl in Sf9 insect cells was recorded with 1024 scans. Processing and analysis of the spectra was performed using the programs XWINNMR (Bruker) and Sparky (T.D. Goddard and D.G. Kneller, SPARKY 3, University of California, San Francisco), respectively.

Results

Selection of the medium

In order to find a suitable medium for selective labeling in insect cells, several media were tested for growth rate and maximal cell density. Table 1 summarizes the characteristics of different widely used media for insect cells, named SF900II, IPL41 and KBM10. SF900II was used as a reference for comparison of the growth rate and maximal reachable cell density. The high contents of amino acids, which were determined by Radford et al. (1997), and glucose in this medium enabled the highest reachable cell density of all examined media. The comparison of the published formulations of IPL41 and KBM10 reveals that the total mass of amino acids in IPL41 is about 100% higher than in KBM10. There are also great differences in the proportion among certain amino acids. Nevertheless nearly identical maximal cell densities were detected for IPL41 and KBM10. Based on these results a reduced content of amino acids was used for the developed media IML406 and IML455. We started with a medium with yeastolate and 5% serum and a second medium with 10% serum but without yeastolate. Whereas the medium with yeastolate enables healthy cell growth after six weeks of adaptation, the medium without yeastolate led to slow growth rates after this time. Based on this result we decided to take the medium with yeastolate as a starting point for studying the influence of serum and yeastolate on the amino acid composition.

Table 1. Comparison of the maximal cell density (N_{max}), the doubling time, the time of exponential growth ($t_{exp.}$) and protein yield (Y) in the media SF900II, KBM10, IPL41 (serum-free and with 5% FCS), IML406and IML455. The yield of GST obtained in SF900II was set to 100%

Medium	N_{max}, $\times 10^6$ ml^{-1}	Doubling time, h	t_{exp}, h	Y
Sf900 II	5.7	20–22	100	100%
KBM10	3.4	22–26	80	60–63%
IPL41	3.1	22–26	80	58–63%
(5% FCS)				
IPL41	3.2	22–26	80	26–31%
IML406	4.2	22–26	80	65–74%
IML455	2.2	24–28	70	43–48%%

Different amounts of amino acids are provided by yeastolate and serum

Table 2 depicts the portions of the total amount of each amino acid supplied by serum and yeastolate. Whereas the content of free amino acids in serum is very low the addition of some amino acid species via yeastolate is very high. The yeast autolysate contains very large amounts of glutamic acid, isoleucine, leucine, phenylalanine and valine. For most of the amino acids the portion provided by yeastolate is below 15%. Because the developed medium should be used for selective labeling of proteins with ^{15}N-amino acids, the separate addition of these amino acids should be the main

Table 2. Overview of the proportions of free ^{14}N-amino acids provided by yeastolate (YF) and serum (FC5)

Amino acid	Portion of ^{14}N-amino acid provided by		Amino acid	Portion of ^{14}N-amino acid provided by	
	YE	FCS		YE	FCS
L-alanine	94%	6%	L-leucine	25.7%	<1%
L-arginine	8.3%	0%	L-lysine	12.9%	<1%
L-asparagine	17.4%	0%	L-methionine	20.9%	<1%
L-aspartic acid	14.9%	<1%	L-phenylalanine	24.5%	<1%
L-cystine	14.1%	<1%	L-proline	5.6%	0%
L-glutamic acid	29.9%	2.6%	L-serine	12.5%	<1%
L-glutamine	0.4%	<1%	L-threonine	30%	<1%
Glycine	7.7%	<1%	L-tryptophane	16.8%	0%
L-histidine	8.8%	<1%	L-tyrosine	12.4%	<1%
L-isoleucine	31.8%	<1%	L-valine	32.2%	<1%

source. For this reason we modified the content of amino acids in such a manner that at least two third of each amino acid, except alanine, stemmed not from yeastolate or serum. The resulting medium was IML406. To investigate the possibility of using ammonium chloride for selective ^{15}N-labeling in insect cells, we used the medium IML455, which contained 5 mM ammonium chloride instead of glutamine and aspartic acid to enhance the formation of glutamine from glutamic acid. In a further step the cell growth and the protein yield of these two media with those of other widely used insect cell media were compared and listed in Table 1. For IML406 the growth studies show a slightly higher maximal cell density when compared to IPL41 and KBM10. The cell density in IML455 is 50% lower than for IML406 and the period of exponential growth is shortened in comparison to the residual media. Except for the slightly prolonged doubling time in medium IML455, the doubling times of the other media are nearly identical. The determination of protein yields with test expressions of GST in different media have also been performed. On the one hand no significant differences in yields could be observed between KBM10, IPL41 with 5% serum and IML406; on the other hand the yield in medium IML455 was more than halved when compared to reference SF900II, whereas in serum-free IPL41 the amount of expressed GST was reduced by 70%. Based on these data the media IML406 and IML455, showing sufficient protein yields, were used for ^{15}N-labeling studies using NMR spectroscopy.

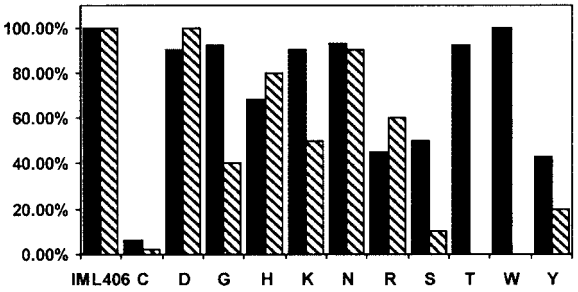

Figure 1. Overview of the maximal cell density (black bars) and the protein yield (hatched bars) in IML406 without separate addition of single amino acids. The values for the complete IML406 were set to 100%. In the case of threonine and tryptophan the protein yields were not determined.

To ascertain if the quantities of certain amino acids provided by serum and yeastolate are sufficient for cell growth to high densities, these compounds were not added separately to the IML406 medium with the consequence that serum and yeastolate were the sole sources for these compounds. A selection of amino acids essential and non-essential for Sf9 insect cells is given in Figure 1. In adhesion cultures no influence on cell growth for all amino acids was seen in the modified IML406 over a period of ten passages, whereas in suspension cultures significant differences were detected, as shown in Figure 1. In the case of cystine no high cell densities and protein yields were obtained. Reduction of arginine, serine and tyrosine led to a decrease of the maximal cell density by 50%. In the medium without the separate addition of histidine the cell density reached 75% of the complete IML406.

The reduction of the other examined amino acids had no negative influence on the insect cells in suspension cultures. Furthermore we performed expression studies with GST in the above mentioned modified media (Figure 1). In the case of tyrosine and serine the yield dropped in a range of 10–20% of the complete IML406. In IML406 containing only half the amount of separately added serine and tyrosine the cell growth and protein yield was equal to the normal IML406 (data not shown). The yields for arginine, glycine and lysine were decreased to 40–65% of the normal values, whereas in the medium without separate addition of histidine the yield was reduced to 80%. For the other investigated amino acids no significant reduction in yields were detected.

^{15}*N-labeling with glycine, lysine, valine, phenylalanine or leucine*

For different labeling studies on GST (Figure 2), the protein was expressed in Sf9 and *E. coli* using single ^{15}N-amino acids. In both expression systems the yields of GST ranged between 8 and 12 mg of the protein per liter of culture. In most cases the quantities of ^{15}N-amino acids utilized in IML406 and the medium for bacterial expression were similar. One exception was lysine that was added to IML406 in an amount three times larger than that in bacterial media. Since the assignment of GST is not available, a comparison of the complete number of resonances, their shape and intensities of the signals should be sufficient to provide qualitative informations about the efficient use of the new media. The starting points for the interpretation were the known metabolism pathways in *E. coli*. Table 3 presents a comparison between the signals seen in the HSQC spectra of selectively labeled GST expressed in Sf9 and *E. coli*. The results of selective labeling of GST with ^{15}N-glycine, ^{15}N-phenylalanine, ^{15}N-valine and ^{15}N-leucine are presented in Figures 3a–d. Selected examples for ^{15}N-amino acids (showing cross labeling) are depicted in Figures 4a–d.

Figure 3a shows the ^{15}N-HSQC spectrum of the purified GST labeled with ^{15}N-glycine in Sf9 and *E. coli*. The spectrum exhibits 17 strong and several weak peaks in case of expression in insect cells. In the spectrum obtained from the bacterial expression system 17 intensive peaks can be identified. 15 identical signals can be found in both spectra. GST expressed in Sf9 contains 16 glycines, GST from *E. coli* 17 glycines. The number of strong peaks in both organisms is close to the number that is expected for glycine. 550 mg l^{-1} serine in IML406 was used, and in the medium for *E. coli* 1.6 g l^{-1}.

HSQC spectra of ^{15}N-lysine labeled GST in insect cells and *E. coli* are very similar (data not shown). The protein possesses 21 lysines and the spectra of both systems show 17 identical resonances. In both spectra the total number of intensive signals is smaller than the number of lysines in GST. For two peaks a difference in chemical shift can be found depending on the host of expression. The differences were not caused by posttranslational modifications since a MALDI-TOFF analysis of GST expressed in Sf9 revealed only the mass of unmodified GST. The higher amount of lysine in IML406 had no influence on the total number of intensive peaks.

For labeling of GST with ^{15}N-valine 200 mg l^{-1} of the amino acid was used for bacterial and viral expressions (Figure 3b). The HSQC spectrum shows 11 strong peaks and additionally some weak peaks in the case of expression in Sf9. The spectrum of bacterially expressed ^{15}N-valine GST contains 14 strong and 7 weak signals. Several strong signals can be assigned to peaks in the ^{15}N-glutamic acid HSQC spectrum (vide infra). Ten resonances are identical in both spectra, which corresponds to the number of valines in GST. For the eleventh signal in the spectrum from Sf9 no corresponding signal from other spectra can be found. For the identification of cross labeling we labeled GST with ^{15}N-alanine in *E. coli*. The HSQC spectrum contains 10 peaks, three less than expected. Seven signals could be assigned to weak peaks in the ^{15}N-valine spectrum recorded with GST expressed in bacteria (Figure 4a). In the case of viral expressed GST these peaks are not visible.

Figure 3c depicts GST labeled with ^{15}N-phenylalanine in *E. coli* and insect cells. GST holds nine phenylalanines. Comparison of both spectra reveals significant differences in the number of signals. Whereas the viral expression of GST leads to nine strong signals, the HSQC spectrum of GST from *E. coli* contains more than 15 strong signals. One strong signal of the HSQC spectrum from Sf9 has no counterpart in the spectrum from *E. coli*. More than ten peaks of the HSQC spectrum derived form bacterially expressed protein are identical with signals in the spectrum of GST labeled with ^{15}N-tyrosine in *E. coli* (Figure 4b). Strong signals from one spectrum correspond to weak peaks in the other spectrum. In IML406 250 mg l^{-1} ^{15}N-phenylalanine were used, in the medium for bacterial expression 100 mg l^{-1}. De-

E. coli MSPILGYWKIKGLVQPTRLLLEYLEEKYEEHLYERDEGDKWRNKKFELGLEFPNLPYYID
Sf9 *MSPILGYWKIKGLVQPTRLLLEYLEEKYEEHLYERDEGDKWRNKKFELGLEFPNLPYYID*

GDVKLTQSMAIIRYIADKHNMLGGCPKERAEISMLEGAVLDIRYGVSRIAYSKDFETLKV
GDVKLTQSMAIIRYIADKHNMLGGCPKERAEISMLEGAVLDIRYGVSRIAYSKDFETLKV

DFLSKLPEMLKMFEDRLCHKTYLNGDHVTHPDFMLYDALDVVLYMDPMCLDAFPKLVCFK
DFLSKLPEMLKMFEDRLCHKTYLNGDHVTHPDFMLYDALDVVLYMDPMCLDAFPKLVCFK

KRIEAIPQIDKYLKSSKYIAWPLQGWQATFGGGDHPPKSDLVPRGSPGIPGSTRAAAS
KRIEAIPQIDKYLKSSKYIAWPLQGWQATFGGGDHPPKSDLVPRGSPGIHRD

Figure 2. Primary sequences of the expressed constructs of GST in *E. coli* and Sf9. The different amino acids in the C-termini are colored in red.

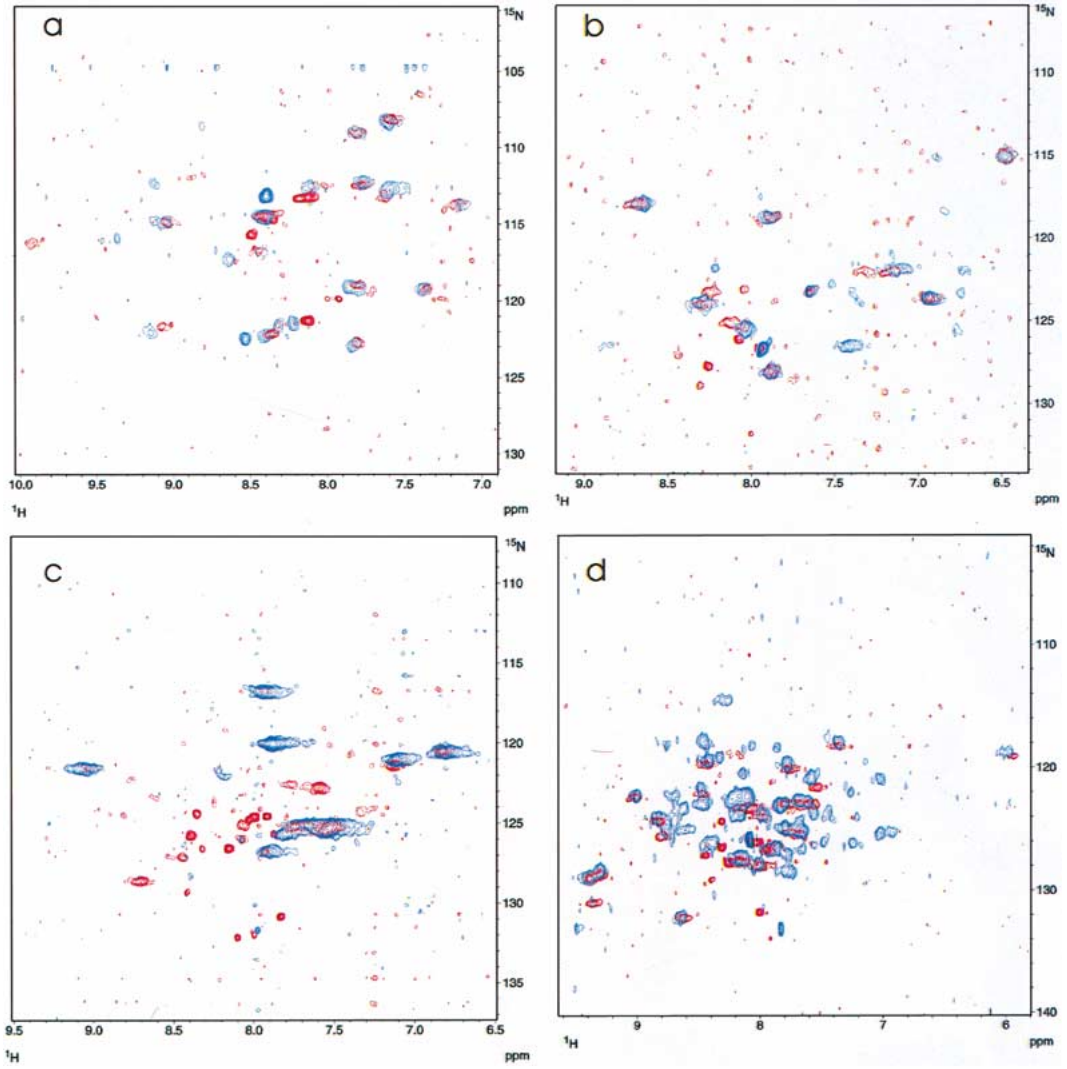

Figure 3. Overlay of ^{15}N-HSQC spectra, recorded with GST expressed in *E. coli* (red) and Sf9 (blue). The protein was selectively labeled with ^{15}N-glycine (a), ^{15}N-valine (b), ^{15}N-phenylalanine (c) or ^{15}N-leucine (d).

Table 3. Comparison of the number of signals obtained in HSQC spectra recorded with GST selectively labeled with ^{15}N-amino acids in Sf9 or *E. coli*. The number of identical signals for a certain amino acid is also listed

^{15}N-amino acid	Number of amino acids in GST expressed in		Number of signals in the GST spectra (strong/weak) from		Number of identical signals (strong/weak)
	E. coli	BEVS	*E. coli*	BEVS	
GLY	17	16	16	17	15
LYS	21	21	19	19/2	17
VAL	10	10	14/7	11/6	10
PHE	9	9	18/6	9/3	8/2
LEU	28	28	24/4	34/10	22
GLU	16	16	50/18	40/4	31
ASP	18	19	49/18	0/0	

spite the higher amount of phenylalanine for Sf9 no conversion to other amino acids was detectable since phenylalanine and tyrosine are essential for insect cells.

We investigated also the possibility of using reduced amounts of certain amino acids for efficient labeling. For this purpose we expressed ^{15}N-glycine labeled GST in IPL41 and we used KBM10 for expression of the ^{15}N-valine or ^{15}N-phenylalanine labeled GST. In the case of the^{15}N-glycine labeling in IPL41 the spectrum showed the same result as in IML406. The usage of KBM10 with ^{15}N-valine or ^{15}N-phenylalanine led to spectra of poor quality. The peaks were at the same protein concentration less intensive (data not shown).

For ^{15}N-leucine (Figure 3d) differences in the number of peaks are visible depending on the expression system. Whereas the bacterial expression of GST led to a spectrum of about 24 strong and several weak signals the expression in Sf9 gave an HSQC spectrum with more than 30 strong peaks. GST contains 28 leucines and 22 peaks can be found in both spectra. We also labeled GST with ^{15}N-isoleucine in *E. coli* and compared the HSQC spectrum with the spectrum of ^{15}N-leucine labeled GST in *E. coli*. Several strong peaks in the spectrum of leucine correspond to weak peaks in the spectrum of isoleucine (data not shown). At least in *E. coli* there exists an efficient conversion from leucine to isoleucine. A comparison of the respective spectra revealed an efficient conversion of isoleucine to valine in *E. coli*. In insect cells these reactions are not visible. But the spectrum of GST labeled with ^{15}N-leucine in Sf9 contains still a large number of signals, which could not be assigned to amino acids investigated so far.

^{15}*N-labelling with ammonium chloride, glutamic acid or aspartic acid*

It was of major interest to investigate the possibility of using ammonium chloride for ^{15}N-labeling of proteins in Sf9. The spectrum contained two groups of signals in a region that is typical for amides in the side chains of glutamine or asparagine (data not shown). In other regions of the HSQC spectrum no peaks were visible. Especially the incorporation of ^{15}N-nitrogen in alanine reported by Drews et al. (2000) was not detectable even after recording the HSQC spectrum with 1024 scans.

For completeness ^{15}N-glutamic acid (Figure 4c) and ^{15}N-aspartic acid was use for labeling of GST in Sf9 and *E. coli*. In the bacterial expression system both amino acids led to HSQC spectra with more than 50 signals. GST contains 18 aspartic acids and 16 glutamic acids. The superposition of both spectra revealed that most of the strong signals were identical. An overlay of the HSQC spectra derived from ^{15}N-isoleucine, ^{15}N-leucine, ^{15}N-phenylalanine, ^{15}N-tyrosine and ^{15}N-valine with the ^{15}N-glutamic acid HSQC spectrum from *E. coli* showed that a large number of strong signals in these spectra correspond to weak or strong signals in the ^{15}N-glutamic acid spectrum. Furthermore, the HSQC spectrum of GST labeled with ^{15}N-glutamic acid contained a number of resonances, which could be identified as cross labeling to alanine by superposition of respective HSQC spectra. In contrast to these results the labeling of GST with ^{15}N-glutamic acid in insect cells led to a spectrum

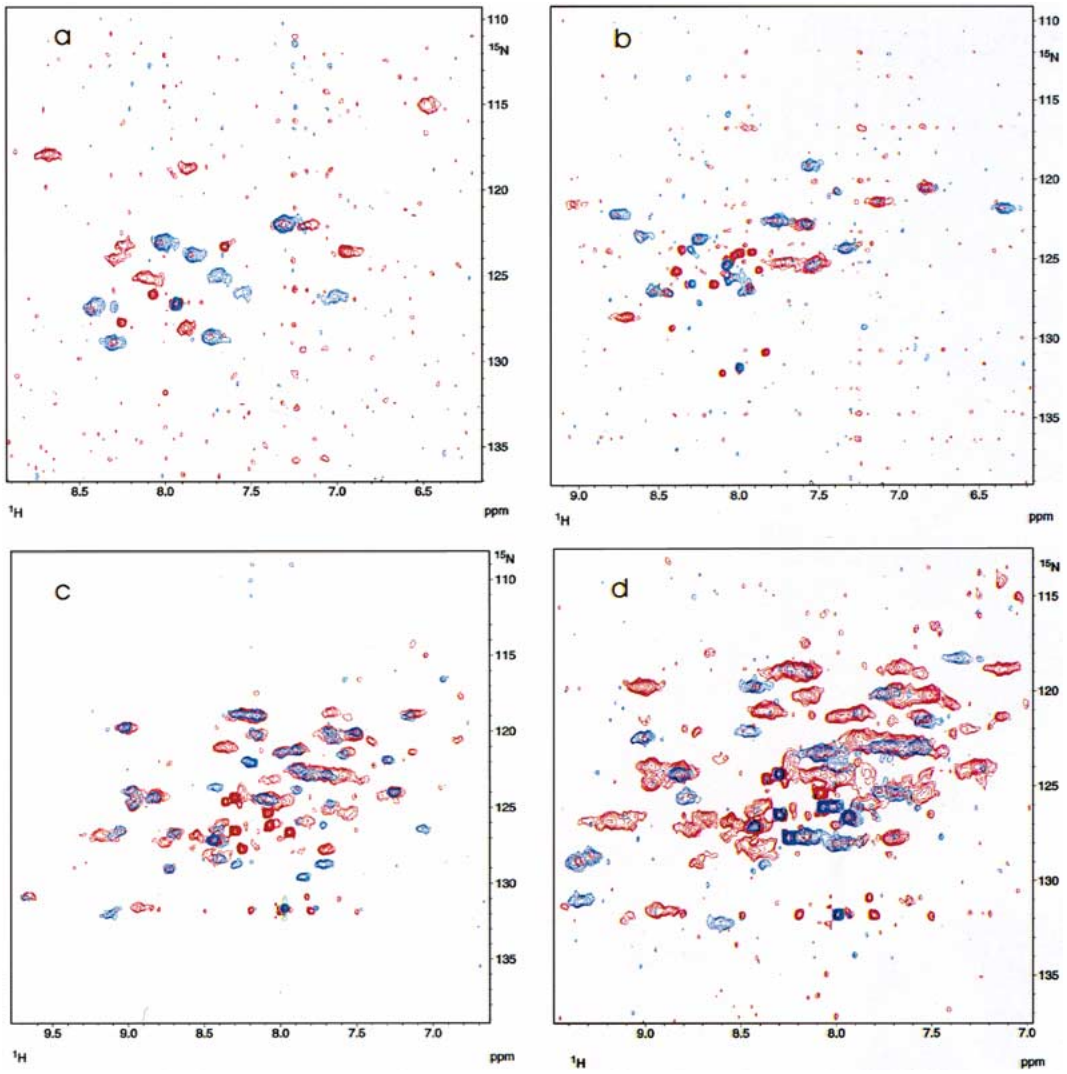

Figure 4. Selected examples of amino acids showing cross labeling in *E. coli* and Sf9: (a) Superposition of HSQC spectra of GST labeled with ^{15}N-valine (red) and ^{15}N-alanine (blue) in *E. coli*; (b) overlay of GST labeled with ^{15}N-phenylalanine (red) and ^{15}N-tyrosine (blue) in *E. coli*; (c) overlay of the HSQC spectra of GST labeled with ^{15}N-glutamic acid in *E. coli* (red) and Sf9 (blue); (d) superposition of the HSQC spectra of GST labeled with ^{15}N-glutamic acid (red) and ^{15}N-leucine (blue) both in *E. coli*.

with only 40 strong and 4 weak peaks. The comparison with the HSQC spectrum of GST derived from *E. coli* exhibited a high conformity of the strong signals in both spectra. Seven signals in the spectrum of viral expressed GST were identified as alanines. In contrast to the expression in bacteria, cross labeling to isoleucine, leucine (Figure 4d), phenylalanine, tyrosine or valine is not detectable. In the case of labeling with ^{15}N-aspartic acid in Sf9 no interpretable spectrum could be obtained.

Finally we compared the HSQC spectra of selectively labeled GST expressed in insect cells with the spectrum of the uniformly ^{15}N-labeled GST expressed in *E. coli* (Figure 5) using ^{15}N-ammonium chloride as sole nitrogen source. Almost all strong peaks are identical in both spectra. Only four signals of the viral expressed GST have no equivalent resonances in the uniformly labeled GST. Some of them are present in the spectra of selectively labeled GST expressed in *E. coli*. Also, most of the weak signals are expected to originate from the protein. A large fraction of the peaks in ^{15}N-GST can be assigned to a certain type of amino acid using the HSQC spectra of selectively labeled GST expressed in insect cells.

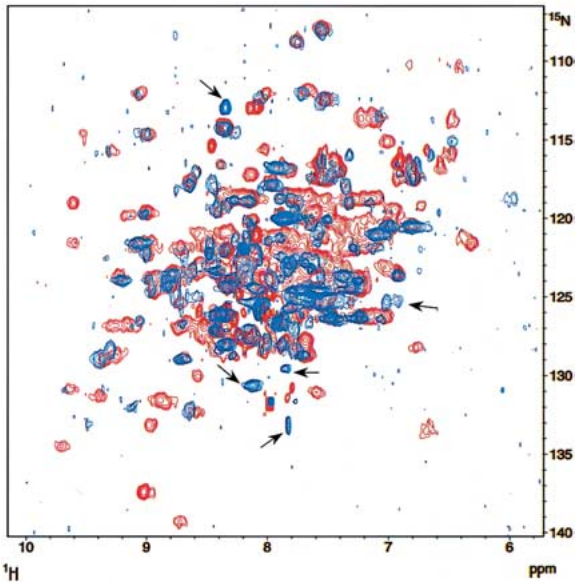

Figure 5. Comparison of HSQC spectra of selectively labeled GST expressed in Sf9 insect cells (blue) with the HSQC spectrum of uniformly ^{15}N-labeled GST expressed in *E. coli* (red). Strong signals, which are not found in the spectrum of ^{15}N-GST, are marked with arrows.

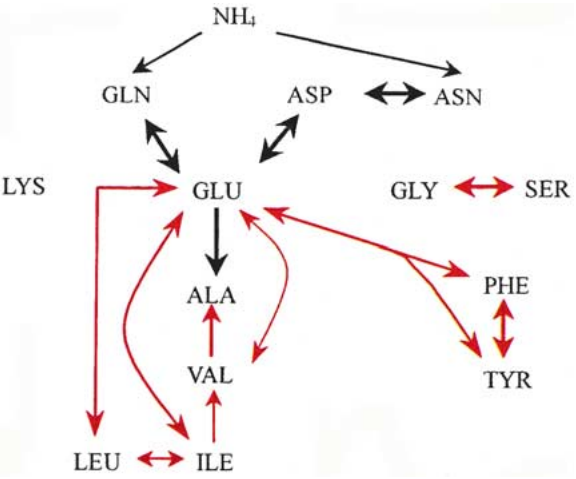

Figure 6. A simplified presentation of the amino acid metabolism in *E. coli* and Sf9 in respect to ^{15}N: The black arrows symbolize pathways which are present in both organisms; the pathways that only exist in *E. coli* are shown in red. The strength of the arrows reflects the intensity of the conversion.

Discussion

For protein-ligand binding studies or for structure determination with NMR spectroscopy on proteins expressed in insect cells the use of selectively labeled protein samples is essential. A medium for the selective ^{15}N-amino acid labeling in insect cells has to fulfill several criteria: (1) The quantities of amino acids should be reduced to a minimum for high cell densities and protein yield. (2) If possible, the use of serum or yeastolate should be decreased without negative influence on protein yields. (3) The formulation should be known and available for the community. Whereas uniformly labeled media are available from several companies, the possibility for obtaining selectively labeled media is restricted. The formulation of a medium, which can be flexibly utilized for selectively labeling, is therefore highly desired. The medium developed here, IML406, has almost the same behavior with respect to growth rate and protein yield as purchasable media or other commonly used insect cell media like, for example, IPL41. The reduced amount of amino acids compared to IPL41 or SF900II has no negative influence on protein yield. No cost-intensive feeding strategies have to be used.

Influence of free amino acids provided by serum and yeastolate

Most of the amino acids are essential for insect cells. This means the conversion of amino acids into each other plays a minor role in Sf9 compared to *E. coli*. It is possible to obtain a high degree of isotopic labeling for an essential amino acid if its separate addition is the major or the sole source in the medium. Media for insect cells usually contain, however, yeastolate and/or serum. Both components provide free amino acids in different quantities. For most of the amino acids the portion of free amino acids supplied by yeastolate or serum was below 15% or 1% of the total mass, respectively. Therefore, the degree of ^{15}N-incorporation should be high enough to obtain interpretable HSQC spectra. Some amino acids (e.g., valine or leucine), which are often used for selective ^{15}N-labeling in *E. coli*, are present in high amounts in yeastolate. We were forced to increase the amount of these amino acids added separately to the medium. The consequence is an increase of costs for labeling experiments with these amino acids. One solution to prevent this is the reduction of yeastolate. To investigate the influence of the amounts of certain amino acids provided by serum and yeastolate on cell growth and protein yield we did not add these amino acids to IML406 separately. If cell growth and protein yield is not influenced by this modified medium the essential amino acid is provided in sufficient quantities by serum and yeastolate. In the case of nonessential amino acids the cells are able to produce these compounds in suf-

ficient amounts. In media without separate addition of arginine, serine or tyrosine the maximal cell density was one half to that of a complete IML406. This indicates that the quantities of these amino acids provided by serum or yeastolate is low (8%, 12% and 12% of the total mass for arginine, serine or tyrosine, respectively). The cells exhausted the essential amino acids arginine, serine and tyrosine very fast and stopped dividing. For serine and tyrosine a reduction of protein yields to 10–20% is observed compared to the complete IML406. The amounts of these amino acids added with the virus solution is not sufficient to enable high protein yields. For serine and tyrosine a high degree of ^{15}N-labeling is therefore possible. In the case of arginine we obtained about 60% of the yield of the complete IML406. Arginine was provided in higher amounts by the virus stock. Under the experimental conditions maximal concentration of arginine was 35 mg l^{-1} in the medium after infection. This represent only 5% of the total amount of this amino acid in the complete IML406. For this reason the amount of ^{14}N-arginine provided by the virus stock can be neglected. The halving of arginine, serine or tyrosine in the medium did not reduce the maximal cell density and the protein yield. Therefore the labeling experiments can be performed with reduced amounts of these amino acids. In IML406, without separate addition of histidine, the maximal cell density reached 70%, the protein yield 80% of the normal IML406. Histidine is provided in small amounts by serum and yeastolate (9% of total mass) and consequently indicates only a small consumption of histidine by Sf9 insect cells. This finding is in agreement with experimental results that showed an increase of histidine in the insect cell medium in the first 80 h of cultivation (Radford et al., 1997). For efficient ^{15}N-labeling the added quantities of ^{15}N-histidine used here are therefore sufficient. In the case of glycine, lysine, threonine and tryptophan the maximal cell density was not influenced when compared to the complete IML406. The amino acids were provided by serum and yeastolate in sufficient quantities (8%, 13%, 30% and 17% of total mass for glycine, lysine, threonine and tryptophan in IML406, respectively) to enable high cell densities. Media without separate addition of glycine and lysine yielded a 50 to 60% reduced protein expression. The separate addition of these amino acids was necessary to obtain high protein yields and a high degree of ^{15}N-incorporation. Expression studies for threonine or tryptophan were not performed so far but the degree of ^{15}N-labeling will be reduced in the case of threonine when the high amount provided by yeastolate is considered. In the case of leucine the quantity delivered by yeastolate is sufficient since in medium KBM10 over 70% of the amino acids stems from this component. For labeling, however, the separate addition of this amino acid is necessary. Aspartic acid, asparagine and glutamine are not essential for Sf9 insect cells. The growth and expression studies show that the cells are able to synthesize these amino acids in sufficient quantities. In the case of the nonessential amino acid cystine, it was not possible to reach high cell densities and protein yields in suspension culture within 100 h. These results correspond to findings of Öhman et al. (1996). The reason may be that the insect cells reach the stationary state after inoculation resulting in a prolonged lag phase and decreased growth rates and maximal cell density. The addition of 80 mg l^{-1} ^{15}N-cystine should be sufficient to obtain a high degree of isotopic labeling, since only 14% of the total mass stemmed from yeastolate. In general, the amount of free amino acids provided by yeast extract can be eliminated, since insect cells can grow in media without the yeast extract but they have to be supplemented with 10% (v/v) dialyzed serum (Gibbs et al., 1992; Öhman et al., 1996; Mitsuhashi, 1982). The adaptation of the cells to a medium without yeastolate is time consuming and not completed so far. Therefore the initial reduction of yeastolate to 2 g l^{-1}, which would decrease the portions of each ^{14}N-amino acid at least below 20% of the total mass, would be an alternative. In the case of amino acids, which were provided by yeastolate in high amounts, a new adjustment of amounts may be necessary using growth studies. It is also possible to eliminate the addition of free amino acids during viral infection since the virus particles can be isolated from the virus stock via precipitation. In this way ^{15}N-incorporation of over 95% is guaranteed for essential amino acids.

Selective labeling with ^{15}N-amino acids

The selective labeling with ^{15}N-amino acids in Sf9 resulted in most of the cases in an equal or better quality of the NMR spectra when these were compared to the spectra obtained from GST expressed in bacteria. Even in the cases were the amount of the ^{15}N-amino acid was only two third of the total mass we obtained interpretable HSQC spectra. With one exception the used amount of ^{15}N-amino acids was sufficient for selective ^{15}N-labeling. The number of scans used for acquisition of HSQCs was similar for

most of the investigated amino acids in both expression systems and therefore, the ^{15}N-incorporation is similar using the bacterial medium and IML406. In some cases of bacterially expressed GST we were forced to use a lower number of scans to be able to distinguish between expected and cross labeled peaks. This demonstrates the high efficiency of amino acid conversion in *E. coli*. In several HSQC spectra of GST derived from bacterial expression very sharp signals were identified. They stem probably from impurities or flexible residues. Since most of the amino acids are essential for insect cells, a conversion between these amino acids is therefore not possible. Since the assignment of GST is not available, we compared the number of total peaks and determined the number of identical peaks in the spectra obtained for one ^{15}N-amino acid. In a first approach we consider only strong peaks as provided by this amino acid in its ^{15}N-version. Weak peaks were considered as formed from ^{15}N-amino acids by cross labeling like transaminations. These signals were compared with strong signals in HSQC spectra of GST labeled with other ^{15}N-amino acids. In the first step we focused on metabolism pathways known in *E. coli* (Waugh, 1996; Muchmore et al., 1989). If a high conformity of the signal pattern is visible, we concluded that the weak signals in one spectrum represented cross labeling to an amino acid causing strong signals in the other spectrum. Signals that do not correspond to peaks in other spectra cannot be assigned to a certain type of an amino acid. In a last step we compared the signals of selectively labeled GST with the peaks of ^{15}N-GST obtained using $^{15}NH_4Cl$ as sole nitrogen source. In some HSQC spectra a line broadening was visible which corresponds to the tendency of GST for aggregation.

The labeling with ^{15}N-glycine or ^{15}N-lysine led to nearly identical results in both expression systems and the number of signals was close to the number of both amino acids in GST. One common feature of bacteria is the conversion of glycine into serine and vice versa. In Sf9 insect cells no formation of glycine out of serine is detectable because these cells do not harbor a mitochondrial serine hydroxymethyltransferase (Tremblay et al., 1992). In the case of *E. coli* the total number of peaks indicates that this conversion is suppressed by the excess of ^{14}N-serine in the medium. For ^{15}N-lysine in both expression systems no cross labeling is visible. This amino acid is not efficiently used for formation of other amino acids. Two peaks differ in chemical shift depending on host cell indicating pH-sensitivity of these lysines since the mass spectrum shows no posttranslational modification of GST expressed in insect cells.

The crucial problem of cross labeling in *E. coli* appeared when ^{15}N-phenylalanine and ^{15}N-valine were utilized for selective labeling. Phenylalanine is converted into tyrosine and valine to alanine catalyzed by special aminotransferases in *E. coli* encoded by the genes aspC and tyrB or avtA respectively (Waugh, 1996). These reactions are not detectable in Sf9 since these cells do not harbor the enzymes needed for this conversion. Tyrosine is essential for insect cells and cannot be formed from phenylalanine (Gibbs et al., 1992) and formation of alanine from valine is also not visible. The eleventh peak found in the spectrum of the GST ^{15}N-Phe expressed in Sf9 can be considered as impurity. The labeling studies with valine and phenylalanine using amounts typical for KBM10 led to spectra of poor quality. This can be explained by the fact that the amount of ^{15}N-amino acid dropped to 50% of the total amount of the amino acid. The elimination of yeastolate is a prerequisite before you can reduce the amount of these two amino acids.

The use of ^{15}N-leucine in IML406 led to a HSQC spectrum in which the number of strong signals clearly exceeds the number of leucines in GST. This result is not explainable so far. Further investigations are required for this amino acid, since we found some evidences that the ^{15}N-nitrogen of leucine is used for formation of glutamic acid. In the case of bacterial expression the number of strong peaks is lower and close to the number of leucines in GST. In *E. coli* leucine is used for the formation of isoleucine. Isoleucine is converted in a second step to valine. The last step of the NH amide formation of leucine and isoleucine from the NH of glutamic acid is catalyzed by a branched-chain aminotransferase encoded by ilvE (Waugh, 1996; Muchmore et al., 1989). For that reason some signals correspond to signals in the HSQC spectrum from *E. coli* using ^{15}N-glutamic acid. This kind of enzyme is also present in Sf9 insect cells but both reactions are not detectable in Sf9. The explanation is that Sf9 insect cells are not able to form the respective precursors, the alpha- keto acids.

It was reported that ammonium is used in Sf9 for formation of the amide of alanine and the side-chain NH group of glutamine. Thus ammonium chloride would be a cheap alternative for selective labeling of alanine in insect cells. Our findings confirm the incorporation $^{15}NH_4$ in the amide groups of arginine and glutamine but the formation of ^{15}N-alanine was not detected, even when we used large number of scans

for the HSQC spectrum. The reason may be that the efficient formation of ^{15}N-alanine from $^{15}NH_4$ starts 72–80 h after the beginning of fermentation as reported by Drews et al. (2000). At this point in time the infected cells were already harvested. In both expression systems we detected a powerful conversion between glutamic acid and aspartic acid. In insect cells no interpretable spectrum for GST labeled with ^{15}N-aspartic acid could be obtained. The reason was the high amount of unlabeled asparagine, glutamine and glutamic acid, which was about seven times higher than the content of ^{15}N-aspartic acid in IML406. The labeling of ^{15}N-aspartic acid is efficiently suppressed. In *E. coli* the fraction of ^{15}N-aspartic acid is higher resulting in an interpretable spectrum. For efficient labeling in insect cells the amount of this amino acid would have to be increased. A better alternative is to remove aspartic acid from the medium and to label it simultaneously with glutamic acid. The higher amount of ^{15}N-glutamic acid in IML 406 led to a spectrum of good quality. A reduction or elimination of yeastolate would enhance the ^{15}N-incorporation. The formation of alanine from glutamic acid is better visible in Sf9 than in bacteria indicating a more efficient pathway in insect cells. Since only seven out of the total ten alanines in GST expressed in Sf9 were visible the remaining three were not detectable due too weak signal intensity. In contrast to the medium for bacterial expression IML406 contains no additional alanine and the cross labeling is not suppressed. Identical to the bacterial expression system addition of 400 mg l^{-1} ^{14}N-alanine to IML406 will reduce the intensity of the peaks originating from ^{15}N-alanine. The total number of peaks in the spectrum of GST labeled with ^{15}N-glutamic acid in Sf9 was only lower by four resonances when compared the total number of aspartic acid, glutamic acid and alanine in GST. Therefore these three amino acids can be labeled efficiently using ^{15}N-glutamic acid. No cross labeling to other amino acids was visible. *E. coli* use glutamic acid additionally to form many other amino acids like valine, phenylalanine, leucine or tyrosine. The transamination between these amino acids plays a central role in bacteria since they harbor many aminotransferases, which use glutamic acid as donor for amino groups. In Sf9 this reaction is limited to amino acids involved in the citric acid cycle and alanine. Using ^{15}NH4Cl and ^{15}N-glutamic acid, glutamic acid, glutamine, aspartic acid, asparagine and alanine can be labeled simultaneously in IML406.

The final comparison of the HSQC spectra of GST selectively labeled in Sf9 with uniformly ^{15}N-labeled GST expressed in *E. coli* showed that most of the very weak signals in the spectra of selectively labeled protein can be considered as impurities. Only four strong signals did not correspond to peaks of ^{15}N-GST indicating impurities, too.

Based on our findings we propose in Figure 6 a simple and provisional overview of the network of the amino acid metabolism in *E. coli* and insect cells focused on nitrogen. We show that the transamination is limited to few reactions in insect cells, whereas in *E. coli* the nitrogen is transferred between most of the amino acids. Especially the central role of glutamic acid for synthesis of amino acids in *E. coli* is clearly visible.

Figure 6 also shows that the expression in Sf9 is better suited for selective labeling of amino acids. The expression system offers the possibility to selectively label amino acids to high degrees and without cross labeling for tyrosine, phenylalanine, glycine, serine, cysteine, arginine and valine. Even amino acids can be labeled that are normally not used in *E. coli* due to extensive cross labeling. After elimination of yeastolate from IML406 the degree of ^{15}N-incorporation in Sf9 will be higher than in *E. coli*. The expression in insect cells is then in many cases a potential alternative to the in-vitro-translation. This is important for proteins that are not available in high yields or in a functional form in bacteria. In addition selective labeling in Sf9 using all essential amino acids could accelerate the assignment of NMR signals for proteins which can be expressed in bacteria or insect cells to comparable yields.

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