Identification of the tRNAs which up-regulate agrostin, barley RIP and PAP-S, three ribosome-inactivating proteins of plant origin

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Abstract Ribosome-inactivating proteins (RIP) are RNA-*N*-glycosidases widely diffused in plants which depurinate ribosomal RNA at a specific universally conserved position, A4324 in rat ribosomes. A small group of RIPs (cofactor-dependent RIPs) require ATP and tRNA to reach maximal activity on isolated ribosomes. The tRNA which stimulates gelonin was identified as $tRNA^{Trp}$. The present paper reports the identification of three other tRNAs which stimulate agrostin ($tRNA^{Ala}$), barley RIP ($tRNA^{Ala}$, $tRNA^{Val}$) and PAP-S ($tRNA^{Gly}$), while for tritin-S no particular stimulating tRNA emerged. The sequences of $tRNA^{Val}$ and $tRNA^{Gly}$ correspond to the already known ones (rabbit and man, respectively). The $tRNA^{Ala}$ (anticodon IGC) identifies a new isoacceptor. Only the stimulating activity of the $tRNA^{Ala}$ for agrostin approaches the specificity previously observed for the couple gelonin-$tRNA^{Trp}$.

Key words: Ribosome-inactivating protein; tRNA; Agrostin; Barley ribosome-inactivating protein; Gelonin; PAP-S; Tritin-S

1. Introduction

Ribosome-inactivating proteins (RIPs), although also active on DNA substrates [1–4], are best known as RNA-*N*-glycosidases which inactivate ribosomes by specifically removing a single adenine from a highly conserved loop present in the large RNA of the large ribosomal subunit (for review see [5]). While most RIPs are quite efficient in inactivating highly purified isolated ribosomes, some do require help from cofactors [6–8]. Cofactor-dependent and cofactor-independent RIPs can even be present in different parts of the same plant where probably they have a different role and function [8].

All RIPs up to now identified as cofactor-dependent require both ATP and macromolecular components of the post-ribosomal supernatant for maximal activity on isolated ribosomes [6,9]. One of the macromolecular components of the supernatant responsible for the up-regulation of RIPs has been identified as tRNA [9,10]. Only in the case of gelonin, the RIP from *Gelonium multiflorum*, the active tRNA has been isolated and sequenced [10]. The evidence collected points to mammalian $tRNA^{Trp}$ as a highly specific cofactor for gelonin.

The results reported in the present paper definitively indicate, as previously suggested [9], that the nature of the tRNA involved and the relative role played by ATP and tRNA as stimulators of RIPs vary with different RIPs. The tRNAs which stimulate three cofactor-dependent RIPs, agrostin (from *Agrostemma githago* seeds), barley RIP (from *Hordeum vulgare* seeds) and PAP-S (from *Phytolacca americana* seeds), have been isolated and sequenced.

2. Materials and methods

2.1. Chemicals

Agrostin and gelonin were purchased from Sigma. PAP-S and barley RIP were a generous gift of Professor F. Stirpe of our Department. Tritin-S (from wheat germ) was prepared as previously described [7]. Tryptophanyl-tRNA synthetase was prepared from beef pancreas [11] and tRNA nucleotidyl transferase from rabbit reticulocyte lysate [12]. [8-^{14}C]ATP (51 mCi/mmol), [^{3}H]tryptophan (30 Ci/mmol) and other radiochemicals were from Amersham.

2.2. Purification of rabbit tRNAs

Total rabbit tRNA was isolated from livers by classical phenol extraction procedures and differential precipitations [13]. The pure tRNA species were purified from total tRNA in three steps: two successive column chromatographies on benzoylated DEAE-cellulose (BD-cellulose) and Sepharose 4B, and, as third step, a two-dimensional polyacrylamide gel electrophoresis (2D-PAGE). All these procedures, as well as the elution conditions of the tRNAs from PAGE, have been reported previously [14–16].

2.3. Primary structure determination of tRNAs

To sequence the tRNAs and to characterize the modified nucleotides therein we used ^{32}P-postlabelling procedures as already described [14,16]. These involved: (i) single hit random hydrolysis of the purified tRNAs; (ii) T4 polynucleotide kinase 5′ ^{32}P-postlabelling of the resultant fragments according to their length; (iii) separation of the postlabelled fragments; (iv) analysis by thin layer chromatography of the end nucleotides of each fragment after total hydrolysis with P1 nuclease and electrophoresis-homochromatography through those regions which could not be determined directly by end nucleotide analysis; (v) analysis of the end oligonucleotides by T1 RNase or by pancreatic RNase digestions of those fragments where hypomodifications were expected. The latter analysis included separation of the oligonucleotides on DEAE-cellulose paper, high voltage electrophoresis in 7% formic acid and determination of their radioactive 5′ end nucleotides for the accurate assignment of hypomodification rates of partially modified positions.

2.4. Trimming of the 3′-CCA end of tRNAs

Stepwise removal of the 3′ terminal nucleotide was performed chemically according to Paulsen and Wintermeyer [17]. One, two or three cycles of the periodate-aniline-phosphatase treatment (Whitfeld cycles) yield, respectively, tRNAs missing the 3′-terminal A, or CA, or the whole CCA tail. The product of each trimming cycle was checked by measuring the 3′ end labelling with [^{14}C]ATP catalyzed by tRNA nucleotidyl transferase [12]. The assays, performed in the absence and in the presence of CTP (0.13 mM), gave the predicted results.

2.5. Assay of the RIP-stimulating activity of tRNA

This was performed as previously described [7,9]. Briefly, 80S *Artemia salina* ribosomes (10 pmol in 10 μl) were preincubated with the RIP in the absence and in the presence of tRNA. RIPs were used at

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concentrations that were completely ineffective (0.8 nM agrostin, 5 nM gelonin, 4 nM barley RIP, 1 nM PAP-S, 5 nM tritin-S) or only slightly effective (see legend to Fig. 3) in the absence of tRNA. Unless otherwise indicated, 1 mM ATP was present in the assays in order to maximize the stimulating effect of tRNAs for all RIPs [9]. After 10 min at 28°C, 2.5 μl samples were withdrawn and the extent of inactivation of ribosomes was quantitated in a 100 μl poly(U) translation system. In this system the RIP from the preincubation step is made ineffective by the extensive dilution.

3. Results

3.1. Purification and identification of the RIP stimulator tRNAs

In the first step rabbit tRNA was fractionated on BD-cellulose. The stimulating activities for agrostin, barley RIP, PAP-S and tritin-S rose steeply at the emergence of the optical density eluted by the NaCl gradient, then declined in the case of agrostin and barley RIP, while remaining more sustained for PAP-S and even more so for tritin-S. This behavior is consistent with the spreading of the stimulating activities for PAP-S and tritin-S previously observed with unfractionated tRNA electrophoresed on 10%-PAGE in the presence of 4 M urea [9]. On the same column the stimulating activity for gelonin eluted later and separated in two peaks. Amino acylation performed with [^{3}H]tryptophan and tryptophanyl-tRNA synthetase showed that the first peak, eluted at 0.9 M NaCl, contained $tRNA^{Trp}$ and the second, requiring ethanol for elution and stripping at pH 9 for amino acylation, tryptophanyl-tRNA. These results are consistent with the identification of $tRNA^{Trp}$, in both the uncharged and charged form, as a highly specific cofactor for gelonin [18].

The fractions from BD-cellulose containing most of the stimulating activities for agrostin, barley RIP, PAP-S and tritin-S were pooled and further purified on Sepharose 4B. The stimulating activity for all four RIPs was mostly present in the peak with higher optical density eluted at 1.45 M ammonium sulfate, although some activity clearly spread also in other fractions.

In the third and last purification step, the material of this peak was subjected to 2D-PAGE (Fig. 1). While the stimulating activity for tritin-S was distributed throughout the spots, precluding the identification of a specific tRNA, the activity

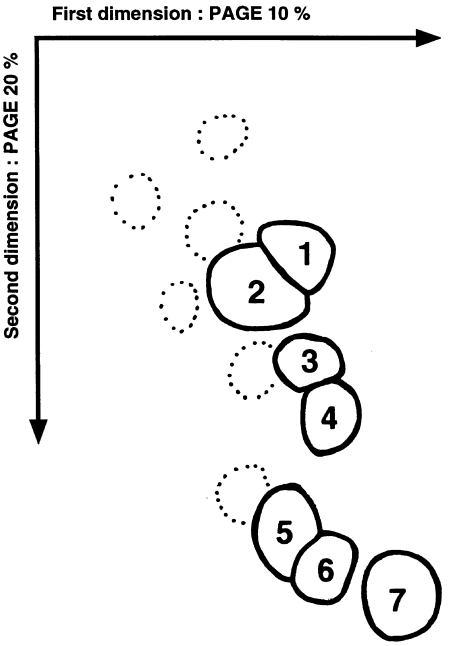

Fig. 1. 2D-PAGE of tRNAs from Sepharose 4B. The numbered spots were assayed for the different RIP-stimulatory activities (for results see text).

for agrostin was maximal in spot 1, that for PAP-S in spot 7 and that for barley RIP was equally present in spots 1 and 2. The sequence of the tRNA stimulating agrostin identified a new isoacceptor $tRNA^{Ala}$ (anticodon IGC) differing from the already known ones (human $tRNA^{Ala}$, A9990 or A9991 in compilation [19]) by 18–20 nucleotides, respectively. Among the differences, 10–12 nucleotides belong to base pairs that are compensatorially changed between these isoacceptors. The other differences are located in the D, T and extra loops. Interestingly, two differences must be pointed out: these concern (i) the so called invariant T that is present in our tRNA whereas the other already known $tRNA^{Ala}$ from higher organisms [19] carry an A at that position like in the eukaryotic initiator tRNAs [19] and (ii) the absence of m^1A in position 58 that could signify that the present isoacceptor $tRNA^{Ala}$

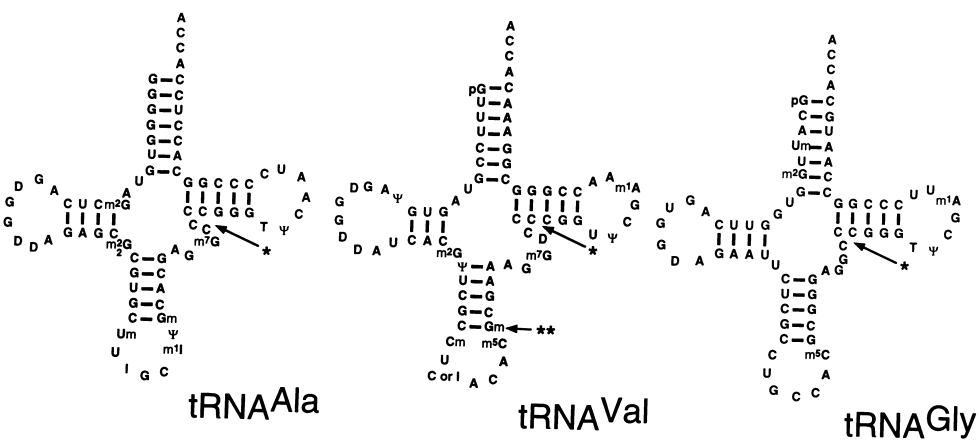

Fig. 2. Cloverleaf drawing of the RIP-stimulating tRNAs from spots 1, 2 and 7 of 2D-PAGE: $tRNA^{Ala}$ (spot 1); $tRNA^{Val}$ (spot 2) and $tRNA^{Gly}$ (spot 7). *One or more of these C_s are methylated, m^5C_s. Their positions have not been determined. **G_m in that position is hypomodified.

does not carry the m^1A methyl transferase recognition elements.

The stimulating activity for barley RIP was shared by the $tRNA^{Ala}$ and by a tRNA carrying the anticodons IAC and CAC identical to the already published $tRNA^{Val}$ from rabbit liver (V9220 in compilation [19]). Finally a tRNA identical to the already known human $tRNA^{Gly}$ (anticodon GCC, G9991 in compilation [19]) stimulated PAP-S. The sequences of the three tRNAs from spots 1, 2 and 7 are shown in Fig. 2.

3.2. *Effect of ATP and of the integrity of the -CCA ends of tRNAs on the stimulating activities*

Rabbit tRNAs have mainly an intact -CCA end as resulted from the primary structure deduction of several tRNAs, including the above mentioned ones, and from analytical PAGE, performed in semi-denaturing as well as denaturing conditions, where pure species behaved as single bands. In contrast, lamb tRNAs show an important loss of 3′ end nucleotides: A or CA. With these tRNAs we always found duplication, and even triplication of the bands on PAGE. A degradation of the 3′ terminus was expected since the livers were obtained at the slaughterhouse and 1 h at least elapsed before extraction of tRNA.

On BD-cellulose the agrostin-stimulating activity of lamb tRNA eluted in the same position as rabbit tRNA. However, 10-fold less tRNA was required to stimulate agrostin (0.8 nM) to give 50% inhibition of protein synthesis.

This increase in stimulating activity prompted experiments in which the -CCA tail of the rabbit tRNA from BD-cellulose was progressively trimmed of the 3′ terminal nucleotide. As shown in Table 1, removal of the terminal A increased 10-fold the stimulating activity of the tRNA, while further trimming had little effect. The presence or absence of 3′P was insignificant.

An increase in the stimulating activity of tRNA for agrostin after trimming of the terminal A was also observed with the tRNA eluted from Sepharose 4B. Instead the trimming did not increase, but actually depressed the stimulating activities of the tRNAs for barley RIP, PAP-S and tritin-S (data not shown). Further points of similarity between agrostin and gelonin thus emerge: (i) the stimulating effect of tRNA is only poorly improved by the presence of ATP [9] and (ii) with both RIPs trimming of the -CCA tail increases the stimulating activity of tRNA ([18] and present data). In contrast,

Table 1
Effect of trimming of the CCA end of rabbit tRNA from BD-cellulose on the stimulating activity for agrostin

	tRNA required for 50% inactivation of ribosomes (ng)
tRNA	101.1
tRNA (minus A)	10.0
tRNA (minus pA)	9.4
tRNA (minus CpA)	8.7
tRNA (minus pCpA)	8.0
tRNA (minus CpCpA)	13.6
tRNA (minus pCpCpA)	11.3

Trimming of the pooled fractions from BD-cellulose was performed as described in Section 2. At each Whitfeld cycle, the agrostin-stimulating activity of the trimmed tRNA was assayed before and after the phosphatase treatment. The amount of tRNA required for the inactivation of 50% ribosomes was calculated by linear regression between fractional activity of ribosomes and log of the amount of tRNA. 2 nM agrostin was present.

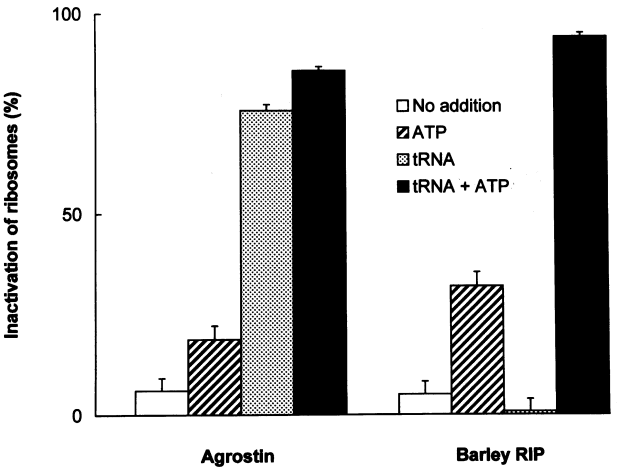

Fig. 3. Different effect of ATP and tRNA on the inactivation of ribosomes by agrostin and barley RIP. Agrostin was 2 nM, barley RIP 15 nM, ATP 1 mM and tRNA (from BD-cellulose) 300 ng. Bars are S.D. ($n=3$). The mean dpms ($\pm$S.D., $n=6$) incorporated in the absence of RIPs were: no addition 11 543 ± 474; ATP 11 177 ± 379; tRNA 11 351 ± 432; tRNA and ATP 11 704 ± 335.

in the case of barley RIP, PAP-S and tritin-S, the presence of ATP and an intact -CCA end in tRNA are required for maximal stimulating activities.

The different effect of ATP on the stimulating activities of tRNAs for the various RIPs is exemplified in Fig. 3 which compares the effect of the two cofactors, added singly and together, on agrostin and barley RIP. In the experimental conditions chosen, ATP alone, although more active on barley RIP than on agrostin, only moderately increased the inactivation of ribosomes by both RIPs. With agrostin, tRNA was highly effective and the simultaneous addition of ATP had at most an additive effect. With barley RIP, the presence of ATP stimulated a concentration of tRNA by itself completely ineffective to give a population of fully inactivated ribosomes. A possible explanation of the results may lie in the different specificity of the requirement for tRNA, a more stringent one in the case of agrostin, and a broader one, for at least two and possibly more tRNAs, in the case of barley RIP. The interaction of a specific tRNA with its target (ribosome or RIP or their complex) may not require an additional factor such as ATP, while in the case of a less stringent specificity ATP could force tRNAs with lower affinity to participate to the process. The integrity of the -CCA end of tRNAs may have a role in this effect of ATP.

4. Discussion

The search for RIP-stimulating tRNAs has led to the purification from rabbit liver of three tRNAs which stimulate agrostin ($tRNA^{Ala}$), barley RIP ($tRNA^{Ala}$ and $tRNA^{Val}$) and PAP-S ($tRNA^{Gly}$). All along the purification procedure no particular tRNA emerged with stimulating activity for tritin-S.

Comparing the data for gelonin [9,10] with those obtained for the four RIPs in the previous [9] and present paper a spectrum of tRNA dependence emerges. Gelonin is specifically stimulated by $tRNA^{Trp}$. In contrast, the stimulatory activities for agrostin, barley RIP and PAP-S can be traced to one (agrostin and PAP-S) or two (barley RIP) particular

tRNAs, but other tRNAs are not completely devoid of such activity. The spreading of the activity among different tRNAs is particularly noticeable in the case of tritin-S.

RIPs are used in anti-viral and cancer therapy as immunotoxins with the antibody moiety targeted to specific markers on the unwanted cells (see [5]). The success of an immunotoxin depends on the choice of the antibody and also on that of the toxic moiety, the 'ideal' toxin requiring little non-specific toxicity and a great efficacy against the target cells. In the case of cofactor-dependent RIPs, the presence in the undesired cells of an increased level of a specific cofactor tRNA might direct a rational and aimed choice of the RIP to be used. Recent results support this approach. $tRNA^{Trp}$, which is the specific cofactor for gelonin, is also the primer of the reverse transcriptase of Rous sarcoma virus [20] and accumulates in transformed cells [21]. Gelonin proved more active in inhibiting protein synthesis by lysates of the RSV-transformed cells than untreated controls and the increased activity correlated with the content in $tRNA^{Trp}$ (submitted for publication). The results reported in the present paper suggest that, besides the increase of a particular tRNA, also the whole pattern of the various tRNAs in an undesired cell may be relevant for the stimulation of a cofactor-dependent RIP. The appearance, in both virally infected and neoplastic cells, of new isoacceptor tRNAs has long been known [22,23]. These tRNAs are often the result of post-transcriptional hypomodifications or even hypermodifications [24].

The physiological role of RIPs in plants is still poorly understood. The two major hypotheses, a defensive mechanism against viral infections and a role of RIPs in the regulation of cell metabolism, have recently been discussed at length [4], as well as the possible role of cofactor tRNAs in such mechanisms [8]. Such a role requires, however, that plant tRNAs display the same stimulating activity for RIPs as the mammalian tRNAs studied in the earlier and present papers. Although only a few sequences of plant tRNAs are known, they are largely conserved within the plant kingdom and not very dissimilar from those of mammalian tRNAs. Not taking into account post-transcriptional modifications, the tRNAs of *Triticum aestivum*, one of the few plants for which a large number of tRNAs have been sequenced, differ from cytoplasmic mammalian tRNAs with the same anticodon by a minimum of 8 ($tRNA^{Trp}$) to a maximum of 23 ($tRNA^{Arg}$) bases. These differences are smaller than that observed between the two mammalian tRNAs stimulating barley RIP ($tRNA^{Ala}$ and $tRNA^{Val}$ differ from each other by over 30 bases). A RIP-stimulating activity of plant tRNAs similar to that observed with mammalian tRNAs can thus be easily envisaged.

The stimulating activities of the tRNAs specific for gelonin and agrostin share a low dependence on ATP and a large increase in activity after trimming of the -CCA tail. Both tRNAs have in the acceptor arm a repeated sequence of four consecutive G, present in the 5′ side in the case of the $tRNA^{Ala}$ (agrostin) and in the 3′ side in the case of $tRNA^{Trp}$ (gelonin). This sequence is missing in $tRNA^{Val}$ and in $tRNA^{Gly}$ and also in the $tRNA^{Trp}$ from yeast which are completely devoid of gelonin-stimulating activity (present data and [18]). Studies on the correlation between structure and function will take advantage of the recent observation (unpublished data) that synthetic tRNAs, devoid of modified bases, are active in stimulating RIPs.

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References

[1] Barbieri, L., Gorini, P., Valbonesi, P., Castiglioni, P. and Stirpe, F. (1994) Nature 372, 624.
[2] Barbieri, L., Valbonesi, P., Gorini, P., Pession, A. and Stirpe, F. (1996) Biochem. J. 319, 507–513.
[3] Stirpe, F., Barbieri, L., Gorini, P., Valbonesi, P., Bolognesi, A. and Polito, L. (1996) FEBS Lett. 382, 309–312.
[4] Barbieri, L., Valbonesi, P., Bonora, E., Gorini, P., Bolognesi, A. and Stirpe, F. (1997) Nucleic Acids Res. 25, 518–522.
[5] Barbieri, L., Battelli, M.G. and Stirpe, F. (1993) Biochim. Biophys. Acta 1154, 237–282.
[6] Sperti, S., Brigotti, M., Zamboni, M., Carnicelli, D. and Montanaro, L. (1991) Biochem. J. 277, 281–284.
[7] Carnicelli, D., Brigotti, M., Montanaro, L. and Sperti, S. (1992) Biochem. Biophys. Res. Commun. 182, 579–582.
[8] Carnicelli, D., Brigotti, M., Alvergna, P., Pallanca, A., Sperti, S. and Montanaro, L. (1997) J. Exp. Bot. 48, 1519–1523.
[9] Brigotti, M., Carnicelli, D., Alvergna, P., Pallanca, A., Sperti, S. and Montanaro, L. (1995) FEBS Lett. 373, 115–118.
[10] Brigotti, M., Carnicelli, D., Alvergna, P., Pallanca, A., Lorenzetti, R., Denaro, M., Sperti, S. and Montanaro, L. (1995) Biochem. J. 310, 249–253.
[11] Kisselev, J.L., Favorova, O.O. and Kovaleva, G. (1979) Methods Enzymol. 59, 234–257.
[12] Brigotti, M., Carnicelli, D., Sperti, S. and Montanaro, L. (1994) Biochem. Mol. Biol. Int. 32, 585–596.
[13] Fournier, M., Dorizzi, M., Sarger, C. and Labouesse, J. (1976) Biochimie 58, 1159–1165.
[14] Keith, G., Pixa, G., Fix, C. and Dirheimer, G. (1983) Biochimie 65, 661–672.
[15] Keith, G. and Heyman, T. (1990) Nucleic Acids Res. 18, 703–710.
[16] Keith, G. (1990) in: Chromatography and Modification of Nucleosides (Gehrke, C.W. and Kuo, K.C., Eds.), Chromatography Library Series Vol. 45A, pp. A103–A141, Elsevier, Amsterdam.
[17] Paulsen, H. and Wintermeyer, W. (1984) Eur. J. Biochem. 138, 117–123.
[18] Brigotti, M., Carnicelli, D., Alvergna, P., Pallanca, A., Sperti, S. and Montanaro, L. (1996) Biochem. Mol. Biol. Int. 40, 181–188.
[19] Sprinzl, M., Steegborn, C., Hübel, F. and Steinberg, S. (1996) Nucleic Acids Res. 24, 68–72.
[20] Harada, F., Sawyer, R.C. and Dahlberg, J.F. (1975) J. Biol. Chem. 250, 3487–3497.
[21] Esposito, F., Russo, T., Ammendola, R., Duilio, A., Salvatore, F. and Cimino, F. (1985) Cancer Res. 45, 6260–6263.
[22] Mushinski, J.F. and Marini, M. (1979) Cancer Res. 39, 1253–1258.
[23] Hatfield, D., Feng, Y.-X., Lee, B.J., Rein, A., Levin, J.G. and Oroszlan, S. (1989) Virology 173, 736–742.
[24] Dirheimer, G., Baranowski, W. and Keith, G. (1995) Biochimie 77, 99–103.