Enzymic Iodination of Eukaryotic Ribosomal Subunits

CHARACTERIZATION AND ANALYSIS BY TWO-DIMENSIONAL GEL ELECTROPHORESIS

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(Received 9 April 1975)

1. Conditions are described for the enzymic iodination of ribosomal subunits from rat liver. The reaction is relatively insensitive to broad changes in the concentration of KCl, allowing subunits to be studied under conditions which minimize their dimerization. 2. Mixtures of extracted ribosomal proteins were iodinated with ^{125}I , the proteins separated by two-dimensional gel electrophoresis and the radioactivity in each protein was determined. Thus 19 out of 23 of the proteins of the small subunit and 25 out of 33 of the proteins of the large subunit were labelled. Iodination should therefore be a suitable method for studying the topography of the ribosomal proteins of rat liver. 3. When the intact 40S subunit (rather than the extracted mixture of proteins) was iodinated, 18 of the 19 proteins were still labelled. However five of these were labelled less strongly than before. When the intact 60S subunit was iodinated, 17 of the 25 proteins were still labelled, although six of these were labelled less strongly. 4. These results show that in rat liver most of the ribosomal proteins of both subunits are at least partially at the surface of the particles. They are also consistent with the idea that the proportion of the ribosomal proteins in the interior of the particle may be greater for the 60S subunit than for the 40S subunit.

A number of chemical and enzymic agents have been used to explore the topography of ribosomal subunits from *Escherichia coli* (Craven & Gupta, 1970; Chang & Flaks, 1970; Gavrilova & Spirin, 1974). Most of these are rather specific, however, and only react with a few of the 'surface' proteins of the particles (Wittmann & Stöffler, 1972). I wished to use a reagent of wider reactivity to study the topography of eukaryotic ribosomal subunits and decided to try the enzyme lactoperoxidase, which catalyses the iodination of tyrosine residues in several different proteins (Marchalonis, 1969; Phillips & Morrison, 1971).

In preliminary studies with one-dimensional gel electrophoresis I showed that this approach could be applied to ribosomal proteins from rat liver (Leader, 1974). The present paper describes the analysis of the iodinated ribosomal proteins by two-dimensional gel electrophoresis, a method which allows greater resolution and provides a common frame of reference for different workers (Kaltschmidt & Wittmann, 1970). It also describes the general characteristics of the reaction pertinent to the effective iodination of ribosomes under mild conditions and where dimerization of particles is minimized.

While this work was in progress, the iodination of ribosomal subunits from *E. coli* was reported (Miller & Sypherd, 1973a,b; Michalski *et al.*, 1973; Litman & Cantor, 1974).

Materials and Methods

Materials

Lactoperoxidase (EC 1.11.1.7) was purchased from Calbiochem, London W.1, U.K., and K^{125}I (carrier-free) from The Radiochemical Centre, Amersham, Bucks., U.K. Other reagents used were generally of analytical grade. Male Wistar rats of 150 g body weight, fed *ad libitum*, were used for all experiments.

Preparation of ribosomes and their subunits

The methods used for preparing ribosomes and their subunits have been described previously (Leader & Wool, 1972). Subunits were concentrated by precipitation with ethanol (Kaulenas, 1971).

Extraction of protein from ribosomal subunits

Proteins were extracted from ribosomal subunits with acetic acid as described by Sherton & Wool (1972).

Sucrose-density-gradient analysis of ribosomal subunits

Analysis was done at 25°C by using linear 15-30% (w/v) sucrose gradients and centrifuging at 234 000g in a Spinco SW50.1 rotor. Times of runs and ionic

conditions are given in the Figure legends. Gradients were examined at 254 nm by using an ISCO gradient analyser.

Two-dimensional gel electrophoresis

The method was that of Kaltschmidt & Wittmann (1970), as adapted for eukaryotic ribosomes (Sherton & Wool, 1972). I used a miniaturized system, similar to that of Howard & Traut (1973), but on an even smaller scale. The apparatus used for the second dimension allowed four 7 cm × 7 cm gel slabs (3 mm thick) to be run simultaneously and was designed and built by Mr. N. Harvey of this department. A similar apparatus is commercially available (e.g. from Pharmacia, Uppsala, Sweden, or Universal Scientific, London, U.K.). The gels used for the first dimension included 6M-urea, 4% acrylamide and 3.3% methylenebisacrylamide (pH 8.7). Ribosomal protein (150 µg) was applied and electrophoresis was for 3 h at 20°C, at 6 mA per gel. The gels used for the second dimension included 6M-urea, 18% acrylamide and 1.4% methylenebisacrylamide (pH 4.5). Electrophoresis was for 15–16 h at room temperature (20°C), at 9 mA per gel. Gels were stained for 2 h at 37°C with 0.1% Coomassie Brilliant Blue in 50% (w/v) methanol–10% (w/v) acetic acid and de-stained electrophoretically for 3 h in 10% (v/v) methanol–10% (v/v) acetic acid.

Iodination of ribosomal proteins

The method used was based on previously published procedures (Marchalonis, 1969; Phillips & Morrison, 1971), adapted as described in the text. Details of individual experiments are found in the legends, but the reaction mixture always contained (per ml): 150 µg of lactoperoxidase, 20 nmol of KI and 1 µmol of Tris-HCl (pH 7.5). The reaction was started by adding H₂O₂ and stopped by adding β-mercaptoethanol to a concentration of 10 mM. The reaction temperature was 20°C.

Analysis of iodinated amino acid residues

The ribosomal proteins were digested with Pronase, and mono- and di-iodotyrosine were separated by paper chromatography as described by Miller & Sypherd (1973a).

Measurement of radioactivity

When the gross radioactivity incorporated into samples of ribosomes was measured, 10% (w/v) trichloroacetic acid was added to precipitate the subunits and these were collected on glass-fibre discs (Whatman GF/C). The discs were placed in vials and the ¹²⁵I was measured in a Beckman Biogamma counter with an efficiency of 80%.

Two-dimensional gels were subjected to radioautography after sealing in polythene bags. Kodak X-ray film (KD54T) was used. The individual stained spots were then cut out by using an extra-fine dissecting scalpel (Clay Adams), placed in counting vials and the ¹²⁵I measured as above. Gamma-counting was especially desirable here to avoid the problem of variable quenching that might arise with scintillation counting.

Results

Characterization of the reaction

Initially I characterized the iodination reaction with 40 S subunits as substrate, essentially similar results (not shown) being later obtained for 60 S subunits. The effect of the amount of 40 S subunits on the extent of the iodination is shown in Fig. 1. From these data it was calculated that about 25 mol of I was incorporated/mol of 40 S subunits (assuming the value of 1.5×10^6 for mol.wt. of the 40 S subunit obtained by Hamilton *et al.*, 1971). Of this 67% was in di-iodotyrosine and 33% in monoiodotyrosine (results not shown). Thus about 16 mol of tyrosine was modified/mol of 40 S subunit. Miller & Sypherd (1973b) obtained a value of 18 mol of tyrosine/mol of 30 S subunit from *E. coli*. With some batches of subunits the extent of the reaction declined

Fig. 1. Effect of the amount of 40 S ribosomal subunits on the extent of their iodination

The amount of 40 S ribosomal subunits indicated was incubated with 0.02 µmol of H₂O₂, 8 µmol of KCl, 0.5 µmol of MgCl₂, and 25 nCi of K¹²⁵I for 15 min in the iodination reaction mixture (0.1 ml). The subunits were precipitated and their radioactivity was determined as described in the Materials and Methods section. The rather high 'zero' value is due to self-iodination of lactoperoxidase and to non-specific adsorption of K¹²⁵I on the acid-precipitated protein.

Table 1. Effect of H_2O_2 concentration on the iodination of 40S ribosomal subunits

Ribosomal 40S subunits (4pmol) were incubated with 8 μ mol of KCl, 0.5 μ mol of $MgCl_2$ and 25 nCi of $K^{125}I$ at the H_2O_2 concentration indicated for 1 min in the iodination reaction mixture (0.1 ml). The subunits were precipitated and their radioactivity was determined as described in the Materials and Methods section.

$[H_2O_2]$ (mM)	I incorporated (nmol)
0	0.036
0.002	0.058
0.02	0.176
0.2	0.182
1.0	0.066

Fig. 2. Effect of incubation time on the iodination of 40S ribosomal subunits

Ribosomal 40S subunits (4pmol) were incubated with 0.02 μ mol of H_2O_2 , 8 μ mol of KCl, 0.5 μ mol of $MgCl_2$, and 25 nCi of $K^{125}I$ in the iodination reaction mixture (0.1 ml) for the time indicated. The subunits were precipitated and their radioactivity was determined as described in the Materials and Methods section. The rather high 'zero' value is due to non-specific adsorption of $K^{125}I$ on the acid-precipitated protein.

at higher concentrations. This was probably due to chemical reduction of the H_2O_2 by residual traces of the β -mercaptoethanol used to prepare the subunits. I have therefore used one of the higher concentrations of H_2O_2 (0.2 mM) consistent with maximal iodination (Table 1). Similar concentrations have been used by other workers (Miller & Sypherd, 1973b; Litman & Cantor, 1974). As the reaction proceeds rapidly, being completed by 2 min at 20°C (Fig. 2), it is possible to minimize the exposure of ribosomes to H_2O_2 .

Subunits iodinated under these conditions co-sediment with unmodified subunits (Fig. 3) and have

Fig. 3. Sedimentation of iodinated 40S subunits on a sucrose gradient

Ribosomal 40S subunits (40pmol) were incubated with 0.2 μ mol of H_2O_2 , 80 μ mol of KCl, 5 μ mol of $MgCl_2$ and 250 nCi of $K^{125}I$ for 2 min in the iodination reaction mixture (1 ml). The reaction was stopped with β -mercaptoethanol and dialysed for 3 h to remove most of the $K^{125}I$ that had not reacted. A tenfold excess of unmodified 40S ribosomal subunits was then added and 200 pmol centrifuged for 1.5 h in a sucrose gradient containing 10 mM-Tris-HCl, 5 mM- $MgCl_2$ and 340 mM-KCl, as described in the Materials and Methods section. The E_{254} (----) and the radioactivity (●) of samples (approx. 0.1 ml) were measured. (The radioactivity at the top of the gradient represents unchanged $K^{125}I$.)

therefore maintained their structural integrity. It would also be interesting to know whether the iodinated ribosomes from rat liver still have functional activity. Unfortunately the commercial preparations of lactoperoxidase used in this work contain an impurity which inactivates ribosomes (Litman & Cantor, 1974). However, it is reassuring to note that similar results for the iodination of *E. coli* ribosomes have been obtained with commercial and more purified preparations of lactoperoxidase (Miller & Sypherd, 1973a; Michalski & Sells, 1974).

When using iodination to identify proteins at the surface of ribosomal subunits, it is desirable to use ionic conditions where the subunits do not dimerize. This is because such dimerization might protect certain specific surface proteins from iodination. I have found that during ultracentrifugation the dimerization of both 40S and 60S subunits occurring at a concentration of 40 mM-KCl was prevented by raising the concentration to 300 mM (results not shown). This is consistent with the results of other workers (Wettenhall & Wool, 1974). It therefore seems preferable to use a high ionic strength for the iodination reaction if this is not inhibitory. Fig. 4 shows that the reaction proceeds well, even at 500 mM-KCl (indeed there may be a small stimulation with increasing salt concentration). I also examined the effect of $MgCl_2$ concentration on the reaction (Fig. 5). Although increasing Mg^{2+} concentration may cause a slight decrease in iodination, the reaction occurs over a wide range of concentrations,

Fig. 4. Effect of KCl concentration on the iodination of 40S ribosomal subunits

Ribosomal 40S subunits (4pmol) were incubated with 0.02 μ mol of H_2O_2 , 0.5 μ mol of $MgCl_2$ and 25 nCi of $K^{125}I$ at the KCl concentration indicated for 1 min in the iodination reaction mixture (0.1 ml). The subunits were precipitated and their radioactivity was determined as described in the Materials and Methods section.

Fig. 5. Effect of $MgCl_2$ concentration on the iodination of 40S ribosomal subunits

Ribosomal 40S subunits (4pmol) were incubated with 8 μ mol of KCl, 0.02 μ mol of H_2O_2 , and 25 nCi of $K^{125}I$ at the $MgCl_2$ concentration indicated for 1 min in the iodination reaction mixture (0.1 ml). The subunits were precipitated and their radioactivity was determined as described in the Materials and Methods section.

Two-dimensional gel electrophoresis

Having established conditions for iodination of discrete ribosomal subunits, I then analysed the iodinated proteins by two-dimensional gel electro-

Table 2. Comparison of the iodination of 40S ribosomal subunits and extracted 40S ribosomal proteins

The stained spots from the two-dimensional electrophoresis gels shown in Plate 1 were cut out and their radioactivity was determined as described in the Materials and Methods section. The results are expressed as a proportion of the radioactivity in protein S3. Values less than 0.2 were regarded as not significantly higher than the radioactivity found in unstained portions of the gel (=n.s.). Similar results were obtained in two separate experiments.

Protein	Relative iodination of extracted 40S protein	Relative iodination of intact 40S subunit
S2	0.43	0.24
S3	1.00	1.00
S4	0.77	0.45
S5	0.24	0.28
S6	0.60	1.41
S7	0.33	0.45
S8	n.s.	n.s.
S9	n.s.	n.s.
S10	1.85	0.84
S11	0.95	0.27
S13	0.36	0.50
S14	0.96	0.66
S15	0.38	0.30
S16	0.61	0.41
S17	0.49	1.31
S18	0.83	0.69
S19	1.63	0.31
S20	n.s.	n.s.
S22/24	0.43	1.02
S23	0.47	0.95
S25	0.42	0.53
S26	0.37	n.s.
S28	n.s.	n.s.

phoresis. For comparative purposes, I also examined the iodination of a mixture of proteins previously extracted from the ribosomal subunits.

The results obtained when 40S subunits and the protein extracted therefrom were iodinated are shown in Plate 1. The numbering system used for the stained proteins (a and b) is that of Sherton & Wool (1972, 1974). (This seems justified by the general similarity between my patterns and theirs and allows me to compare our results. Minor discrepancies are, of course, possible.) Radioautographs of the gels are shown in Plate 1(c) and 1(d). Some qualitative similarities and differences can be seen; however, only the more highly labelled proteins are visible in these radioautographs as further exposure for the less radioactive proteins caused a marked decrease in overall definition. Quantitative data are given in Table 2, where the radioactivity in each protein has been normalized to that of protein S3, which is quite highly and similarly labelled in both cases. A number of points emerge from these quantitative data. First,

EXPLANATION OF PLATE I

Two-dimensional gel electrophoresis of iodinated 40S ribosomal proteins

Ribosomal 40S subunits (4 μ mol) or 40S ribosomal proteins (2mg) were incubated with 3 mmol of KCl, 50 μ mol of MgCl₂, 2 μ mol of H₂O₂ and 25 μ Ci of K¹²⁵I for 2 min in the iodination reaction mixture (10ml). The protein was extracted (in the case of the 40S subunit) and 150 μ g was subjected to electrophoresis and radioautography as described in the Materials and Methods section. (a) Iodinated 40S protein, Coomassie Brilliant Blue stain; (b) iodinated 40S subunit, Coomassie Brilliant Blue stain; (c) iodinated 40S protein, radioautograph; (d) iodinated 40S subunit, radioautograph.

EXPLANATION OF PLATE 2

Two-dimensional gel electrophoresis of iodinated 60S ribosomal proteins

The protocol for this experiment was similar to that described in the legend to Plate 1 except for the use of 60S protein or 60S ribosomal subunits. (a) Iodinated 60S protein, Coomassie Brilliant Blue stain; (b) iodinated 60S subunit, Coomassie Brilliant Blue stain; (c) iodinated 60S protein, radioautograph; (d) iodinated 60S subunit, radioautograph.

Table 3. Comparison of the iodination of 60S ribosomal subunits and extracted 60S ribosomal proteins

The stained spots from the two-dimensional electrophoresis gels shown in Plate 2 were cut out and their radioactivity was determined as described in the Materials and Methods section. The results are expressed as a proportion of the radioactivity in protein L6. Values less than 0.2 were regarded as not significantly higher than the radioactivity found in unstained portions of the gel (=n.s.). Similar results were obtained in two separate experiments.

Protein	Relative iodination of extracted 60S protein	Relative iodination of intact 60S subunit
L3	0.79	0.97
L4	0.70	n.s.
L5	0.47	n.s.
L6	1.00	1.00
L7	0.51	0.24
L8	0.56	0.87
L9	0.20	0.27
L10	0.29	0.32
L11	0.30	0.20
L12	0.29	0.73
L13	0.31	0.66
L14	n.s.	n.s.
L15	n.s.	n.s.
L16	n.s.	n.s.
L17	1.06	n.s.
L18	n.s.	n.s.
L19	0.49	n.s.
L20	n.s.	n.s.
L21	1.04	0.65
L22	n.s.	n.s.
L23	0.31	0.34
L24	n.s.	n.s.
L25	0.27	n.s.
L26	0.58	0.85
L27	0.60	n.s.
L28	0.52	0.23
L29	0.46	0.21
L30	0.26	n.s.
L31	0.25	0.20
L32/33	0.60	0.20
L34	n.s.	n.s.
L35	0.41	n.s.
L36	0.49	0.21

completely unequivocal.) This implies that in the 40S subunit at least 18 proteins are at the surface, although five of these proteins may be more 'buried' than the others. Four of the protein spots (S6, S17, S22/24 and S23) appeared to be relatively more highly labelled in the intact subunit. It is possible that this is due to a change in the conformation of the protein causing an increased exposure of tyrosine residues. Such a change in conformation and susceptibility of tyrosine residues to iodination has been reported for cytochrome *c* (Morrison *et al.*, 1974), and Litman *et al.* (1974) found an increased iodination of certain subunit proteins after combination to form a 70S ribosome.

The results obtained when 60S subunits and protein extracted therefrom were iodinated are shown in Plate 2 and Table 3 (in this case the labelling is compared with that of protein L6). Again the quantitative data show that the majority of extracted proteins (25 out of 33) are labelled and thus may be examined by iodination. (Proteins L14, L15, L16, L18, L20, L22, L24 and L34 are unlabelled.) When these iodinated proteins were compared with those iodinated in the intact 60S subunit it could be seen that eight proteins (L4, L5, L17, L19, L25, L27, L30 and L35) were no longer labelled (proteins L4, L17 and L27 were highly labelled in the isolated 60S protein). In six other protein spots (L7, L21, L28, L29, L32/33 and L36) the extent of iodination was decreased by about 50% or more. Thus, although at least 17 of the 60S subunit proteins are at least partially at the surface, the results are consistent with the idea that a greater proportion of proteins may be 'buried' within the 60S subunit than in the 40S subunit.

Discussion

If enzymic iodination is to be useful for exploring the structure of the ribosome most of the ribosomal proteins must have accessible tyrosine residues. This is so for the ribosomal proteins from rat liver, as 19 out of 23 proteins of the small subunit and 25 out of 33 proteins of the large subunit were iodinated. The present work does not consider the proteins S12, S21, S27, S29, S30 and S31, which I observed only on heavily loaded gels, the protein S1, which I have not observed, and it does not distinguish between proteins S22 and S24. Nor does it consider the proteins L38 and L39, which I observed only on heavily loaded gels, the proteins L1, L2, L37, L40 and L41, which I have not observed, and it does not distinguish between proteins L32 and L34.

In this work I have assumed that those ribosomal proteins labelled in the intact subunits are, at least partially, at the surface of the particles and conclude that this is the case for the majority of proteins examined. Similar conclusions have been drawn for the

significant labelling of 19 of the 23 protein spots occurs when the extracted protein is used as substrate. Hence many of the proteins of the 40S subunit can be examined by iodination, exceptions being S8, S9, S20 and S28. When the intact subunit is used as substrate, iodination of all of these except S26 still occurs. However the labelling of proteins S2, S4, S10, S11 and especially S19 was decreased by about 50% or more. (It seems prudent to restrict comment to those changes in iodination of twofold or greater, which are

ribosomal subunits of *E. coli* from studies using lactoperoxidase (Miller & Sypherd, 1973b; Litman & Cantor, 1974) or antibody fragments (Wittmann & Stöffler, 1972). Thus it appears that in this respect the topography of ribosomal subunits from rat liver resembles that of ribosomal subunits from *E. coli*, despite the greater number and size of eukaryotic ribosomal proteins. There are few other topographic studies of eukaryotic ribosomes, using two-dimensional gel electrophoresis, with which to compare the results presented here. One piece of relevant work is that of Ventigmiglia & Wool (1974), who have identified the proteins of the small ribosomal subunit phosphorylated by protein kinases *in vitro* (although at salt concentrations where dimerization does occur). They find that at least eight proteins may be phosphorylated in the intact 40S particle. These are S2, S3, S6, S10, S14/15, S17, S19 and S25, all of which I found to be iodinated (Table 2).

The interpretation of the decreased iodination of certain proteins of the intact subunit is more difficult. It is quite possible that in such proteins the tyrosine residues available for iodination are masked by other ribosomal proteins in the intact subunit, and an area of the protein, free from tyrosine, is exposed. In addition, conformational changes in the opposite direction to those already mentioned might occur; tyrosine residues which are on the surface of an individual protein may orientate themselves towards the interior of the protein after assembly of the subunit. It cannot therefore be concluded that unlabelled proteins are necessarily buried. Thus although my results are consistent with the situation in *E. coli*, where a greater proportion of proteins are 'masked' in the larger subunit, my data do not allow identification of these 'interior' proteins.

I thank Mrs. J. McWattie for cheerful technical assistance, Dr. A. Volkers for introducing me to lactoperoxidase-catalysed iodination, and Professor R. M. S. Smellie for support through a grant from the Cancer Research Campaign.

References

- Chang, F. N. & Flaks, J. G. (1970) *Proc. Natl. Acad. Sci. U.S.A.* **67**, 1321-1328
- Craven, G. R. & Gupta, V. (1970) *Proc. Natl. Acad. Sci. U.S.A.* **67**, 1329-1336
- Gavrilova, L. P. & Spirin, A. S. (1974) *FEBS Lett.* **39**, 13-16
- Hamilton, M. G., Pavlovec, A. & Peterman, M. L. (1971) *Biochemistry* **6**, 2585-2591
- Howard, G. A. & Traut, R. R. (1973) *FEBS Lett.* **29**, 177-180
- Kaltschmidt, E. & Wittmann, H. G. (1970) *Anal. Biochem.* **36**, 401-412
- Kaulenas, M. S. (1971) *Anal. Biochem.* **41**, 126-131
- Leader, D. P. (1974) *Acta Biol. Med. Ger.* **33**, 685-689
- Leader, D. P. & Wool, I. G. (1972) *Biochim. Biophys. Acta* **262**, 360-370
- Litman, D. J. & Cantor, C. R. (1974) *Biochemistry* **13**, 512-518
- Litman, D. J., Lee, C. C. & Cantor, C. R. (1974) *FEBS Lett.* **47**, 268-271
- Marchalonis, J. J. (1969) *Biochem. J.* **113**, 299-305
- Michalski, C. J. & Sells, B. H. (1974) *Eur. J. Biochem.* **49**, 361-367
- Michalski, C. J., Sells, B. H. & Morrison, M. (1973). *Eur. J. Biochem.* **33**, 481-485
- Miller, R. V. & Sypherd, P. S. (1973a) *J. Mol. Biol.* **78**, 527-538
- Miller, R. V. & Sypherd, P. S. (1973b) *J. Mol. Biol.* **78**, 539-550
- Morrison, M., Gates, R. E. & Huber, C. T. (1974) *Membr. Transform. in Neoplasia, Miami Winter Symp.* **8**, 33-49
- Phillips, D. R. & Morrison, M. (1971) *Biochemistry* **10**, 1766-1771
- Sherton, C. C. & Wool, I. G. (1972) *J. Biol. Chem.* **247**, 4460-4467
- Sherton, C. C. & Wool, I. G. (1974) *J. Biol. Chem.* **249**, 2258-2267
- Ventigmiglia, F. A. & Wool, I. G. (1974) *Proc. Natl. Acad. Sci. U.S.A.* **71**, 350-354
- Wettenhall, R. E. H. & Wool, I. G. (1974) *Methods Enzymol.* **30**, 186-197
- Wittmann, H. G. & Stöffler, G. (1972) in *The Mechanism of Protein Synthesis and its Regulation* (Bosch, L., ed.), pp. 285-351, North-Holland, Amsterdam.