Acknowledgment

I thank the late Dr. Y. C. Lee for preparing radioactive DNA, Ms. Irene Kuo for preparing *E. coli* DNA, and Dr. Joseph Horwitz for valuable discussions during the preparation of the manuscript.

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Conformational Changes of 30S Ribosomes Measured by Intrinsic and Extrinsic Fluorescence†

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ABSTRACT: The intrinsic tryptophan fluorescence and the fluorescence of *N*-(3-pyrene)maleimide, a covalently bound sulfhydryl-specific extrinsic probe, have been used to study the conformation of the 30S ribosomal subunit of *Escherichia coli*. (a) The tryptophan fluorescence spectrum of the free ribosomal proteins is shifted to shorter wavelengths than that of free tryptophan. When the proteins are incorporated into the organized structure of the ribosome, there is a small additional blue shift and the emission band becomes narrower. In 6 M urea, the spectrum of the proteins, whether free or in the ribosome, becomes identical with that of the amino acid, reflecting exposure of previously shielded tryptophan residues. (b) When magnesium-depleted ribosomes are unfolded at low ionic strength, the tryptophan fluorescence spectrum changes, although circular dichroism shows no change in α-helix content of the proteins. (c) Intrinsic and extrinsic fluorescence were both found to be sensitive to a limited and fully reversible transition that takes place when ribosomes are incubated under conditions that increase their activity in vitro. This suggests that both probes may be of use in monitoring conformational changes that occur under conditions consistent with activity. The kinetics of the concurrent changes in extrinsic fluorescence and aminoacyl-tRNA binding activity were compared. (d) Conditions are described for labeling ribosomes with *N*-(3-pyrene)maleimide without impairing their activity.

The biological role of the ribosome calls for it to participate in a complex and repeated series of reactions and interactions, during the course of which the particle is believed to go through a cycle of conformational transitions. It is important to develop techniques that can detect such conformational changes, monitor them while they take place, and supply information on the nature of the change. Fluorescence spectroscopy, a sensitive and versatile technique for studying macromolecular conformation, should be useful in all of these respects. The technique has been applied to the ribosome in several different ways (Barenboim et al., 1969; Daya-Grosjean et al., 1972; Lemieux and Gerard, 1973; Hsiung and Cantor, 1973; Pochon and Ekert, 1973; Pochon et al., 1974; Huang and Cantor, 1975; Huang et al., 1975; Gerard et al., 1975; Schechter et al., 1975), but its use in this field is still at an early stage.

In the work described here, we have used two fluorescent probes as indicators of conformational change in the 30S ribosomal subunit of *E. coli:* the intrinsic tryptophan residues of the ribosome and an extrinsic covalently attached probe, *N*-(3-pyrene)maleimide, a specific sulfhydryl reagent that becomes fluorescent only after reacting covalently with a sulfhydryl group (Weltman et al., 1973). We have compared the tryptophan fluorescence of the ribosomal proteins free in

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solution with that of the same proteins in the ribosome, and have examined the effect on tryptophan fluorescence of the extensive distortions of ribosomal structure caused by urea (Spitnik-Elson et al., 1974) and by the unfolding of magnesium-depleted ribosomes (Spirin et al., 1963; Gesteland, 1966; Spitnik-Elson and Atsmon, 1969). We were also able to detect and follow more limited conformational changes that occur during the reversible inactivation and reactivation of the ribosome (Zamir et al., 1971, 1974; Ginzburg et al., 1973). Both probes were found to be sensitive to this transition, which we had previously been unable to demonstrate by other optical techniques.

Materials and Methods

Buffers. (1) 100 mM NH_4Cl, 1 mM magnesium acetate, 10 mM Tris-HCl (pH 7.4); (2) as 1, but KCl instead of NH_4Cl; (3) 350 mM NH_4Cl, 20 mM magnesium acetate, 10 mM Tris-HCl (pH 7.4); (4) 200 mM KCl, 20 mM magnesium acetate, 10 mM Tris-HCl (pH 7.4); (5) 300 mM KCl, 20 mM magnesium acetate, 10 mM Tris-HCl (pH 7.4), 6 mM 2-mercaptoethanol.

Ribosomes and Ribosomal Proteins. 30S ribosomal subunits were prepared from *E. coli* MRE 600 and analyzed for RNA and protein as previously described (Spitnik-Elson et al., 1974). In the NPM[1] experiments, ribosome concentration was estimated from ultraviolet absorbance (Vogel et al., 1969) using published particle weights (Hill et al., 1969). They were free of 50S subunits and contained 60% RNA and 40% protein by weight. If not used at once, they were stored, in buffer 1, in liquid air and were thawed only once before use. Free ribosomal proteins were prepared by the LiCl-urea method (Spitnik-Elson, 1965). The proteins dissolved readily in urea-containing buffers. At the low concentrations employed (40 μg/ml or less), they also gave clear solutions in the absence of urea and remained fully soluble at high salt concentrations (Spitnik-Elson, 1962). At low salt concentrations turbidity sometimes appeared after several hours; fluorescence measurements were, therefore, made within 1 h after the solution was prepared. Tryptophan was determined by the method of Liu and Chang (1971, 1972) using methanesulfonic acid, instead of toluenesulfonic acid.

Urea. Stock solutions of 10 M urea in water, used for buffers that contained 6 M urea, were passed through a mixed-bed ion exchanger (Amberlite MB-3), shaken 2 h with charcoal (Darco 60, 5 g/100 ml), and filtered through acid-hardened paper (Whatman no. 50). The charcoal had previously been repeatedly washed by shaking with water and centrifugation until the wash water was neutral and had then been dried in air. When treated in this way, 10 M urea solutions were neutral and showed the same fluorescence as distilled water.

Labeling of Ribosomes with N-(3-Pyrene)maleimide. NPM was synthesized according to Weltman et al. (1973). 10^{-4} M stock solutions in ethanol were stored in brown ampules at −15 °C and were stable for long periods of time. Before being labeled with the reagent, 30S ribosomal subunits (3 mg/ml in buffer 4 or 5) were heat activated (Zamir et al., 1971) by incubation at 40 °C for 20 or 60 min and then chilled in ice. Any mercaptoethanol present was removed by dialysis in the cold. Ethanolic NPM (0.1 volume) was added, with stirring, to the desired final concentration and stirring was continued in the cold for 1 h. The reaction was terminated by the addition of 1 μl of 2-mercaptoethanol/ml of reaction mixture. The ribosomes were dialyzed overnight in the cold against two or three changes of an appropriate buffer to remove the NPM adduct of 2-mercaptoethanol, and were either used at once or stored in liquid air in small aliquots. No noticeable loss of fluorescence was observed on prolonged storage. The relative amounts of NPM bound covalently by the ribosomes were estimated from the fluorescence of the labeled ribosomes.

Fluorescence Measurements. Fluorescence emission spectra and intensities were measured with a Perkin-Elmer recording fluorescence spectrometer, Model MPF-3L, using a 10-mm cuvette. In order to avoid the heat activation of inactive subunits during the measurements, spectra were routinely recorded in the cold. The cuvette holder was chilled by a circulating coolant at 0–5 °C, and samples were kept in ice until transferred to cuvettes.

For tryptophan fluorescence, excitation was at 290 nm and emission was recorded between 300 and 400 nm, with a 500-nm reading taken as the baseline. Solvent spectra were recorded under identical conditions during each experiment and subtracted graphically. Ribosome concentration was kept below 100 μg/ml (below 40 μg of protein/ml) to minimize the inner-filter effect due to ribosomal RNA (Pesce et al., 1971). To avoid irregularities in the recorder tracing at high sensitivity, a slow pen response and scanning speed were employed. The contribution of Rayleigh scattering was reduced with an emission filter that cuts off 50% of the light emitted at 290 nm. Spectra of standard tryptophan solutions were routinely recorded to provide a rough day to day calibration and allow an approximate comparison of fluorescence intensities observed on different days.

For NPM fluorescence, excitation was at 330 nm and emission intensity was measured at 375 nm, except where a full emission spectrum was required. Ribosome concentration was 3 mg/ml or less. Below 2 mg/ml, fluorescence was proportional to ribosome concentration. There was a small deviation from linearity above this concentration, with the fluorescence yield increasing. When solutions were to be compared with each other, they were at identical ribosome concentrations.

Other Measurements and Assays. Circular dichroism and sedimentation analyses were performed as described previously (Spitnik-Elson et al., 1974), except that the circular dichroic measurements were carried out at 22 °C in a 10-mm cell at a ribosome concentration of 0.1 mg/ml. The activity of 30S subunits was assayed by measuring their capacity to bind [^{14}C]phenylalanyl-tRNA in the presence of poly(uridylic acid) (Zamir et al., 1971). Radioactivity was determined at 83% efficiency with a Packard Tricarb scintillation counter; the samples counted contained about 12–15 μg of ribosomes.

Results

Intrinsic Ribosomal Tryptophan Fluorescence

Tryptophan Fluorescence Spectra of Ribosomes and Ribosomal Proteins. Typical tryptophan fluorescence spectra of 30S ribosomes, purified proteins prepared from them, and free L-tryptophan are shown in Figure 1A. Averaged data from many determinations are presented in Table I ("urea absent"), where the relative positions of the spectra are shown by means of ratios of fluorescence intensity at two wavelengths, so that an increase in the ratio indicates a shift to the blue and a decrease, a shift to the red. The ribosome and protein spectra, which differ appreciably from that of the free amino acid, are similar to each other but are not identical. The ribosome

[1] Abbreviations used are: NPM, *N*-(3-pyrene)maleimide; Tris, 2-amino-2-hydroxymethyl-1,3-propanediol; EDTA, (ethylenedinitrilo)-tetraacetic acid.

TABLE I: Intrinsic Tryptophan Fluorescence of 30S Ribosomes and Ribosomal Proteins, and the effect of 6 M Urea.[a]

	n^b	Emission Max (nm)	Spectral Width at Half-Maximum Emission (nm)	Ratio of Fluorescence Intensity at Two Wavelengths 325 nm / 345 nm	345 nm / 365 nm	325 nm / 365 nm
Urea Absent						
L-Tryptophan	20	352–356	327–387 = 60	0.44	1.08	0.48
30S Ribosomal proteins	7	338–346	315–375[d] = 60[d]	0.80	1.43[d]	1.15[d]
30S Ribosomes[c]	19	338–343	315–370[d] = 55[d]	0.82	1.58[d]	1.30[d]
6 M Urea Present						
L-Tryptophan	4	352–355	328–387 = 59	0.44	1.08	0.47
30S Ribosomal proteins	5	350–356	327–385 = 58	0.46	1.12	0.51
30S Ribosomes[c]	3	351–357	328–387 = 59	0.44	1.07	0.47

[a] Emission spectra were determined in a number of different salt–buffer media at pH 7.4. Concentrations: tryptophan, 2.0 μg/ml; 30S ribosomal proteins, 13.5–27 μg/ml; 30S ribosomes, 25–57.5 μg/ml (protein content = 10–23 μg/ml). [b] Values are the average of n spectra. [c] Includes only ribosomes not previously unfolded and in a medium that preserves the 30S structure. [d] Probability that the difference between the corresponding values for ribosomes and proteins is significant ≧99% (Fisher, 1946). [e] Two media employed: buffer 3 in 6 M urea, and 1 mM Tris-HCl (pH 7.4), 6 M urea. The first would detach 5–8 proteins and the second almost none (Spitnik-Elson et al., 1974).

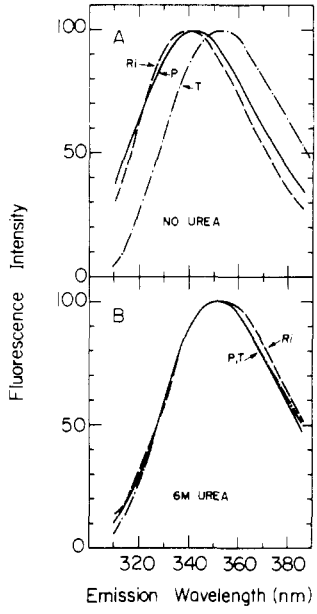

FIGURE 1: Intrinsic tryptophan fluorescence spectra of 30S ribosomes, free ribosomal proteins and tryptophan in the absence and presence of urea. Media: (A) buffer 3; (B) buffer 3 containing 6 M urea. Symbols and concentrations: Ri, ribosomes (A, 57.5 μg/ml; B, 50 μg/ml; contain 23 and 20 μg of protein/ml). P, proteins (A, 13.5 μg/ml; B, 27 μg/ml), T, tryptophan (2.0 μg/ml). All curves are normalized.

spectrum lies slightly to the blue of the protein spectrum and is narrower. The difference is not large but has been confirmed repeatedly.

The Effect of Urea. When 6 M urea was added to the medium, the ribosome and protein spectra shifted to longer wavelengths and became identical with the free tryptophan spectrum, which was not affected by urea (Figure 1B, Table I).

Fluorescence Intensity. We have not made a rigorous comparison of the fluorescence intensities of ribosomes and free ribosomal proteins, but relevant data have accumulated during the course of numerous experiments. Meaningful comparative data were obtained by employing low ribosome concentrations to minimize the inner-filter effect of the ribosomal RNA, which absorbs at the excitation wavelength of 290 nm. At ribosome concentrations below 100 μg/ml, where fluorescence intensity became roughly proportional to concentration, the tryptophan fluorescence intensity of ribosomes approached that of free ribosomal proteins rather closely. The present data are not exact enough to decide whether they are actually equal. On the basis of assays indicating that our preparations contained 5.5–7.5 tryptophan residues per 30S ribosome, the data suggest that tryptophan in the ribosomal protein fluoresces two to three times more intensely than the free amino acid.

Magnesium Ion Depletion and Unfolding. When ribosomes are depleted of Mg^{2+} ions, their structure unfolds increasingly as the ionic strength is lowered, with a concomitant drop in sedimentation velocity (Spirin et al., 1963; Gesteland, 1966; Spitnik-Elson and Atsmon, 1969). The circular dichroic spectrum (Eilam and Elson, 1971, Spitnik-Elson et al., 1974) shows significant structural alterations in the RNA (the spectral region above 240 nm) but none in the proteins (around 220 nm, where the RNA contribution is negligible and the response is due mainly to α-helix structure in the proteins). When Mg^{2+} ions and salt are restored to unfolded ribosomes, their circular dichroic spectrum reverts to that of folded ribosomes (Eilam and Elson, 1971; Spitnik-Elson et al., 1974).

In the experiment described in Table II, folded, unfolded, and refolded ribosomes were prepared and their circular dichroic spectra (data not shown) and sedimentation properties were shown to conform to the behavior described in the preceding paragraph. The effect of these treatments on the intrinsic tryptophan fluorescence is shown in Table II and Figure 2. Unfolding caused a red shift in the emission spectrum and a 30–40% drop in fluorescence intensity. Both changes were reversed on refolding.

Active and Inactive 30S Ribosomes. Urea and magnesium depletion cause large structural changes in the ribosome. In order to test whether tryptophan fluorescence is sensitive to a more limited change, we compared active and inactive forms of the 30S ribosome, which can be easily and reversibly interconverted. It has been shown that the subunits become inactive if the Mg^{2+} ion concentration is reduced to about 1 mM. When the Mg^{2+} concentration is again raised, the subunits rapidly regain activity if heated, and remain active if subsequently chilled. If not heated, they regain activity only very slowly, if at all (Zamir et al., 1971, 1974). The pattern of sulfhydryl reactivity of the ribosomal proteins changes during

TABLE II: Intrinsic Tryptophan Fluorescence of 30S Ribosomes: Effect of Magnesium Depletion and Unfolding.[a]

	Expt	Ribosome concn (μg/ml)	Treatment A (Folded)	B (Folded)	C (Partly Unfolded)	D (Fully Unfolded)	D → A (Refolded)
$s_{20,w}$	1		29.4	29.1		2.6	
	2		29.4	29.8	14.9	2.8	
Relative	1	57.5	100	93	84	57	108
fluorescence	1	28.8	100	95	86	68	
intensity[b]	2	57.5	100	97	95	68	88
	2	28.8	100	109	70	59	
Ratio of	1	57.5	1.42	1.37	1.49	1.22	1.43
fluorescence	1	28.8	1.40	1.35	1.39	1.19	
intensities:	2	57.5	1.35	1.35	1.41	1.30	1.43
325 nm/365 nm	2	28.8	1.29	1.30	1.29	1.03	

[a] A ribosome solution (7–8 mg/ml in buffer 1) was divided into four portions and each was dialyzed with shaking at 2–4 °C for 48 h against 300 volumes of buffer, changed after 24 h. The buffers for the first and second 24-h periods, respectively, were: A, buffer 1, same; B, 1 mM magnesium acetate and 10 mM Tris-HCl (pH 7.4), same; C, 1 mM EDTA and 10 mM Tris, 10 mM Tris; and D, 1 mM EDTA, 10 mM Tris, 1 mM Tris. D → A, refolding was accomplished by mixing a sample of D with more than 100 volumes of buffer 1. Samples, diluted when necessary with the final buffer, were taken for fluorescence, sedimentation, and circular dichroic analysis. [b] Maximum intensities at about 340 nm, normalized to 100 for A.

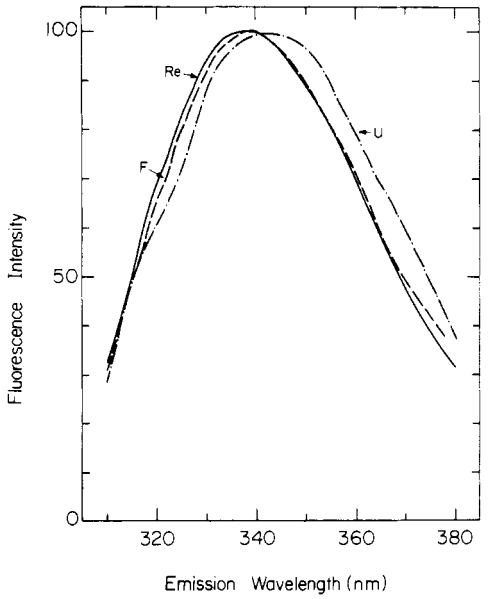

FIGURE 2: Intrinsic tryptophan fluorescence spectra of folded, unfolded, and refolded 30S ribosomes. F, folded; U, unfolded; Re, unfolded and then refolded. The solutions are those of Table II, experiment 2: A, D, and D → A, respectively. See Table II for experimental details.

these transitions, showing that conformational alterations take place (Ginzburg et al., 1973).

In the experiments shown in Table III, inactive subunits were diluted into a cold activating medium and were then heat activated at 40 °C and rechilled. Their fluorescence spectrum was compared with that of a parallel inactive control, an unheated portion of the same diluted ribosome solution. Significant differences were not seen in spectral shape or position. There was, however, a drop of 9–10% in fluorescence intensity when inactive ribosomes were converted to the active form. This drop was not seen when inactive ribosomes were heated in the same way but in a buffer that does not support activation (data not shown), or when they were incubated in the cold in the activating buffer (Table III, expt 1). Thus, the change in

TABLE III: Intrinsic Tryptophan Fluorescence Intensity of Active and Inactive 30S Ribosomes.[a]

Expt	Relative Fluorescence Intensity at 340 nm: Inactive	Active
1		
0 time	100	
20 min, 0 °C	99.0	
45 min, 0 °C	100	
2	100	90.8
3	100	90.5

[a] Inactive ribosomes (7–8 mg/ml in buffer 1) were diluted to a final concentration of 57.5 μg/ml with ice-cold buffer 3, which can support heat activation. A portion of the solution was kept in ice ("Inactive") and another portion was heated 30 min at 40 °C and chilled in ice ("Active"). Fluorescence spectra were recorded within 30 min of this treatment, since inactive ribosomes in buffer 3 may slowly become active in the cold (Spitnik-Elson, unpublished). Fluorescence intensity was maximal at 340 nm in all cases and is normalized to "0 time" (expt 1) or to "Inactive" (expts 2, 3).

fluorescence is due not to the medium or elevated temperature, but to the conformational change that accompanies ribosomal activation.

Fluorescence of the Extrinsic Probe N-(3-Pyrene)maleimide

The reaction of NPM with the 30S Ribosome. The reaction of NPM with the 30S ribosome was found to be closely similar to that of its analogue *N*-ethylmaleimide, which is extensively documented (Traut and Haenni, 1967; Moore, 1971; Ginzburg et al., 1973). Our relevant findings may be summarized as follows. Under the conditions described under Methods, the covalent binding of NPM by ribosomes reached its final level in about 40 min. The uptake of reagent was a linear function of NPM concentration between 10^{-7} and 10^{-4} M, the highest concentration tested. The concentration-dependent uptake of NPM was the same for active and inactive 30S subunits in the

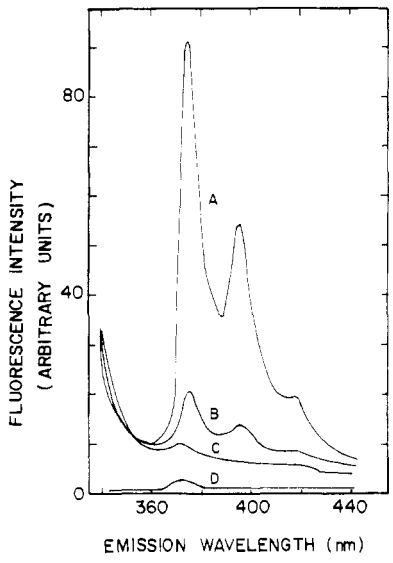

FIGURE 3: The fluorescence spectrum of NPM-labeled 30S ribosomes and the effect of pretreatment with *N*-ethylmaleimide. Ribosomes (3 mg/ml in buffer 4) were heat activated for 20 min and labeled with 1 μM NPM, as described under Methods. For pretreatment, *N*-ethylmaleimide was substituted for NPM, after which the ribosomes were dialyzed against buffer 4 and the procedure was repeated with NPM. Before fluorescence measurements, all samples were dialyzed against buffer 2, ribosome concentration was determined, and all solutions were adjusted to the same ribosome concentration. (A) NPM-labeled subunits; (B) subunits pretreated with *N*-ethylmaleimide and then labeled with NPM; (C) unlabeled subunits; (D) buffer 2 alone.

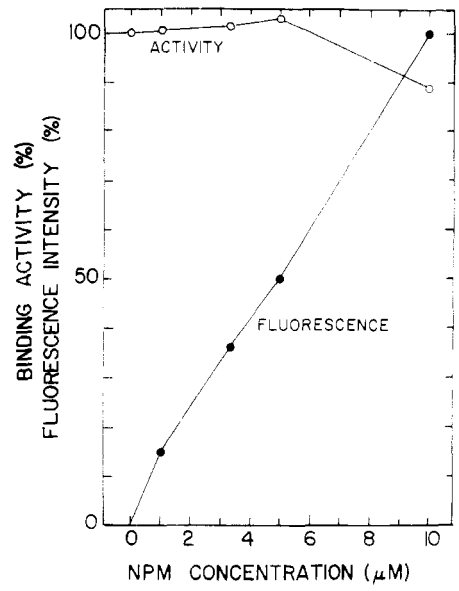

FIGURE 4: Uptake of NPM by 30S ribosomes and its effect on phenylalanyl-tRNA binding activity. 0.5 ml volumes of 30S ribosomes (3 mg/ml in buffer 4) were activated by incubation for 1 h at 40 °C and were labeled with NPM, as described under Methods. After dialysis against buffer 4, duplicate 5-μl aliquots were taken for the assay of binding activity. 0.4 ml was diluted to 2.0 ml with buffer 4 and used for the measurement of fluorescence and ribosome concentration. 100% activity is 135 dpm per μg of ribosomes; samples counted contained 12–15 μg of ribosomes.

presence of 10% ethanol (introduced with the reagent) but in 1% ethanol the uptake by inactive subunits was higher. Active subunits labeled with NPM could be inactivated and reactivated (as described in the preceding section), but activity could not be restored to subunits labeled in the inactive form. Therefore, in order to preserve the ability of the ribosomes to be reactivated and to ensure uniformity of labeling, subunits were always heat activated before being reacted with NPM, as described under Methods.

The fluorescence spectrum of NPM-labeled 30S ribosomes (Figure 3) exhibits two major vibronic bands at about 375 and 395 nm and a shoulder at 415–420 nm. It closely resembles the spectra of several NPM-labeled proteins reported by Weltman et al. (1973), but is shifted slightly to the blue with respect to them.

When ribosomes were treated with the nonfluorescent sulfhydryl reagent *N*-ethylmaleimide before NPM, their fluorescence intensity was diminished by about 85% (Figure 3), indicating that the two reagents compete for the same sites. We have not identified the target protein or proteins of NPM, but a study of fluorescence quenching by iodide ions has indicated that the fluorescent sites on the 30S subunit are heterogeneous. The data obtained with ribosomes labeled in 1 μM NPM yielded a nonlinear Stern–Volmer plot (Stern and Volmer, 1919), whereas a single homogeneous site would give a plot linear with iodide concentration (Lehrer, 1971).

The Effect of NPM on the Activity of 30S Ribosomes. The biological effect of attaching NPM molecules to the 30S subunits was followed by assaying the poly(U)-directed phenylalanyl-tRNA binding capacity of NPM-labeled ribosomes (Figure 4). Ribosomes retained full activity when labeled at NPM concentrations up to 5 μM, where the reaction mixture contained less than two NPM molecules per ribosome. Activity decreased slightly at 10 μM NPM and fell off markedly at still higher concentrations. The experiments described below were carried out with ribosomes labeled, in the active state, at NPM concentrations of 5 μM or less. Under these conditions, activity is not affected, the ribosomes can be inactivated and reactivated, and their fluorescence can be measured without difficulty.

The Effect of Activation on the Fluorescence of NPM-Labeled 30S Ribosomes. The effect of urea and magnesium depletion on the fluorescence of NPM-labeled 30S ribosomes has been reported elsewhere (Schechter et al., 1975). The experiments described here were performed in order to examine the behavior of the extrinsic probe during the heat reactivation of previously inactivated ribosomes, as had been done previously with the intrinsic tryptophan fluorescence (see above). A typical experiment is shown in Table IV, which gives the biological activity and the NPM fluorescence intensity of initially inactive 30S ribosomes before and after heating in buffer 5, which supports heat activation. The heat treatment resulted in an approximately tenfold increase in aminoacyl-tRNA binding activity and a decrease in NPM fluorescence intensity of 20–30%. There was no change in NPM fluorescence when inactive ribosomes were heated in buffer 2, a medium that does not support activation (data not shown).

The kinetics of the heat-induced changes in activity and NPM fluorescence are illustrated in Figure 5. The two parameters did not change at the same rate. Both showed first-order kinetics, but the activity change was faster and proceeded at maximum speed from time zero, as has previously been shown (Zamir et al., 1971), while the slower fluorescence change showed a lag before reaching its maximum rate.

Discussion

The fluorescent behavior of tryptophan in various media is well documented. Its emission maximum is at 350–355 nm in aqueous solution (Teale and Weber, 1957) and shifts to lower wavelengths in less polar solvents (Teale, 1960; Steiner et al.,

TABLE IV: Extrinsic NPM Fluorescence Intensity of Active and Inactive 30S Ribosomes.[a]

NPM Concn During Labeling (μM)	Aminoacetyl-tRNA Binding Activity[b]		Fluorescence Intensity at 375 nm (Arbitrary Units)		
	Inactive	Active	Inactive	Active	% Decrease
1	1.5	25.4	89.5	71.5	20%
3.3	1.9	17.1	155	120	22%
5	2.6	19.1	192	134	30%

[a] A solution of 30S ribosomes (3 mg/ml in buffer 5 lacking mercaptoethanol) was divided into three portions, each of which was labeled with a different concentration of NPM (1, 3.3, or 5 μM) and then inactivated by dialysis against cold buffer 2. Just before the fluorescence measurements, a small volume of concentrated salt–buffer solution was added to convert the medium to buffer 5. The solution was kept in ice until its fluorescence was measured and duplicate samples were taken for the assay of phenylalanyl-tRNA binding activity ("inactive"). The solution was then activated by 1-h incubation at 40 °C and was chilled in ice. Fluorescence was again measured and samples were taken to assay binding activity ("active") and the ribosome concentration was determined. [b] Binding activity is given as the number of phenylalanyl-tRNA molecules bound per 100 ribosomes present. 250 dpm is equivalent to 24 molecules bound.

1964). A similar blue shift, of varying magnitude, occurs when tryptophan is incorporated into proteins, indicating that the amino acid has, to a varying degree, become insulated from the aqueous medium and is in a less polar environment (Teale, 1960; Steiner et al., 1964; Barenboim et al., 1969; Kronman and Robbins, 1970; Burstein et al., 1973). The 30S ribosomal proteins exhibit a considerable blue shift. What is to be noted is that their spectrum undergoes further changes when the proteins are combined with RNA to form the ribosome. The emission peak shifts slightly farther to the blue and becomes narrower, owing to reduced emission at the longer wavelengths. A similar effect has been reported for a single protein, S7, bound to RNA (Gerard et al., 1975).

Exposure to 6 M urea disrupts the structure of the 30S ribosome. The sedimentation coefficient drops to 16–17 S and the circular dichroic spectrum shows extensive destruction of α-helix structure in the proteins (Spitnik-Elson et al., 1974). The fluorescence measurements reported here complement this picture. In the presence of urea, the ribosomal tryptophan fluorescence spectrum underwent a large red shift and became identical with that of the free amino acid. This indicates conformational changes in the proteins that result in the full exposure of previously shielded tryptophan residues to the aqueous solvent (Steiner et al., 1964; Kronman and Robbins, 1970; Burnstein et al., 1973). The effect was the same whether the proteins were free or attached to the ribosomal RNA.

A second treatment used to cause gross distortion of the ribosomal structure was the complete removal of Mg^{2+} ions. Under these conditions, ribosomes unfold when the ionic strength is lowered and their sedimentation coefficient falls (Spirin et al., 1963; Gesteland, 1966; Spitnik-Elson and Atsmon, 1969; see Table II). Circular dichroic spectra have shown the major effect in this case to be on the RNA moiety, with little or no observable change in the α-helix content of the proteins (Eilam and Elson, 1971; Spitnik-Elson et al., 1974). However, a change affecting the proteins can be seen in the tryptophan fluorescence spectrum, expressed as a shift to the red and a decrease in fluorescence intensity. Taken together, the fluorescence and circular dichroic data are consistent with a change in ribosomal conformation that affects the microenvironment but may not greatly disturb the internal structure of individual proteins.

In addition to shifts in spectral position, changes in tryptophan fluorescence intensity were also seen. Although we have not studied them systematically, several observations may be noted. Compared with the free amino acid, the ribosomal tryptophan fluorescence is not only shifted to the blue but is also more intense, by a factor of perhaps two or even more. As mentioned above, the red shift seen when Mg^{2+}-depleted ribosomes are unfolded is accompanied by a decrease in intensity. The fluorescence intensities of the proteins when free or when in the ribosome are similar; we do not know whether they are actually equal.

Unfolding and exposure to urea cause massive structural changes in the ribosome and irreversibly abolish its activity. One of our main reasons for undertaking this work was to attempt to detect smaller conformational changes, closer to those which may take place under physiological conditions. For this purpose, we examined 30S ribosomes whose aminoacyl-tRNA binding activity had been abolished and then restored by relatively mild manipulations of the medium and temperature (Zamir et al., 1971, 1974). The process is fully reversible and the interconversion of the two activity states is accompanied by a structural transition that has been detected as a change in the relative reactivities of the ribosomal sulfhydryl groups (Ginzburg et al., 1973), but could not be discerned in this laboratory by means of optical rotatory dispersion, circular dichroism, or ultraviolet absorption spectroscopy (unpublished results). However, the tryptophan fluorescence was sensitive to this limited structural change, the response being a 9–10% decrease in fluorescence intensity in going from the inactive to the active state. Although this type of measurement yields less detailed information than the sulfhydryl-labeling technique, it has the advantage of being an instantaneous measurement and, in principle, could be used to monitor transitions as they take place in the spectrometer cuvette.

The extrinsic probe NPM was also tested for its ability to detect conformational changes during the activation of initially inactive 30S ribosomes. This reagent, a long lifetime covalently bound sulfhydryl-specific fluorescent probe, was introduced by Weltman et al. (1973), who described its properties and demonstrated its use with a number of proteins. In the present communication, we have described its reaction with the 30S ribosome and its effect on the aminoacyl-tRNA binding activity of the subunit. Activity was found to be impaired if the reaction mixture contained more than two NPM molecules per ribosome, showing NPM to be more inhibitory than its smaller analogue *N*-ethylmaleimide, of which at least seven molecules can be attached per ribosome without affecting activity (Ginzburg et al., 1973). Our experiments were carried out with ribosomes labeled at an NPM level that did not impair activity. The effect of NPM fluorescence of unfolding the ribosome or exposing it to 6 M urea has been reported elsewhere; both treatments caused a marked decrease in fluorescence intensity

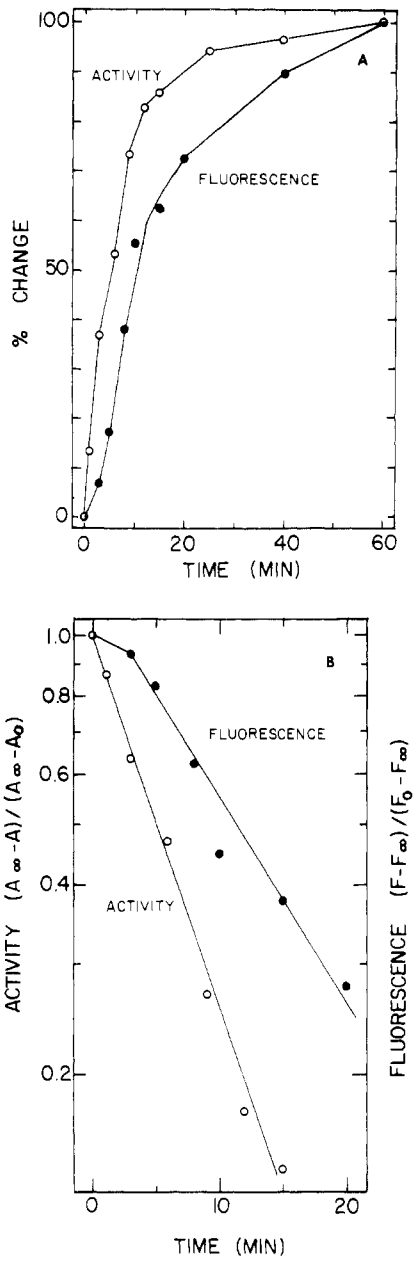

FIGURE 5: Changes in NPM fluorescence during the heat activation of 30S ribosomes. Ribosomes labeled in 5 μM NPM, as in Figure 3 and Methods, were inactivated by dialysis against cold buffer 2. To 6 ml was added 0.5 ml of a concentrated salt–buffer solution to convert the medium to buffer 5. The solution was placed in a 30 °C water bath and aliquots were removed at the specified times for measurement of fluorescence and aminoacyl-tRNA binding activity. For fluorescence, 0.5-ml aliquots were pipetted into 1.2 ml of ice-cold buffer 5. For the binding assay, 20-μl aliquots were pipetted into 25 μl of ice-cold incubation mixture lacking [^{14}C]phenylalanyl-tRNA, which was added later to start the assay. (A) Percent increase in aminoacyl-tRNA binding activity and decrease in fluorescence intensity at 375 nm as a function of time. (B) Semilogarithmic plot of the same data. Symbols: A, activity; F, fluorescence intensity; subscripts: $_0$, 0 time; no subscript, at the time indicated; $_\infty$, after 60-min incubation at 30 °C. First-order rate constants: activity, 0.14/min; fluorescence, 0.08/min.

(Schechter et al., 1975).

The present experiments show that NPM is also sensitive to the smaller conformational changes that take place during the reversible inactivation and reactivation of the 30S subunit. The fluorescence intensity of the ribosome-bound NPM decreased by 20–30% in going from the inactive to the active state. This result shows that NPM fluorescence, like that of tryptophan, can be used to monitor restricted conformational transitions in the ribosome.

For a change in fluorescence to be a valid monitor of a change in activity, the two changes must coincide exactly. This may not occur if the conformational changes that affect activity are accompanied by others that are irrelevant to the activity but influence fluorescence. We investigated this point with NPM-labeled ribosomes. Parallel kinetic measurements showed the fluorescence change to be slower and more complex than the activity change. This indicates that the conformational alterations signaled by NPM fluorescence included one or more alterations not required for the restoration of aminoacyl-tRNA binding sites. Therefore, under the conditions described here, NPM fluorescence can be used to monitor an overall conformational change, but not a change in aminoacyl-tRNA binding activity. Further attempts to achieve a direct correlation between fluorescence and activity should be made with ribosomes in which only a single protein carries the fluorescent probe.

On the whole, the results reported here show that both intrinsic and extrinsic fluorescent probes can be usefully employed to study ribosomal conformation and conformational transitions, including limited ones that take place under conditions consonant with activity.

Acknowledgments

We are grateful to Dr. M. Shinitzky for valuable advice and to Mr. D. Haik for ribosome preparations.

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Dimers and Trimers of Immunoglobulin G Covalently Cross-Linked with a Bivalent Affinity Label†

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ABSTRACT: A bivalent affinity label, bis(α-bromoacetyl-ε-2,4-dinitrophenyllysylproline)ethylenediamine, has been synthesized. Treatment of anti-2,4-dinitrophenyl antibodies with this compound produces a mixture of covalently and noncovalently cross-linked material. Only specific antibodies are covalently cross-linked, suggesting that covalent attachment occurs in the variable regions. Covalently cross-linked dimers and trimers have been isolated from the reaction mixture in a high state of purity, in yields of about 12 and 4%, respectively. The complexes are stable in solutions containing 10^{-4} M hapten and can therefore be used as sensitive probes of immune effector functions.

The interactions of antibodies with antigens provoke a variety of physiological responses (Metzger, 1974), which, in the case of immunoglobulin G (IgG),[1] include activation of the complement system (Müller-Eberhard, 1975; Reid and Porter, 1975), direct binding of antigen–antibody complexes to lymphocytes (Dickler, 1976), and clearance of immune complexes from the circulation (Dorrington and Painter, 1974; Mannik et al., 1971; Waldmann et al., 1971). Antigenic recognition occurs at the N-terminal end of the immunoglobulin molecule (Davies et al., 1975), while the interaction with lymphocytes (Dickler, 1976), macrophages (Unkeless and Eisen, 1975), and components of the complement system (Reid and Porter, 1975) is thought to take place in the Fc portion, some 80 Å away. Two general mechanisms have been proposed (Metzger, 1974) to explain how antigen binding can exert an effect at this distance. According to one proposal, an antigen-induced conformational change within the immunoglobulin molecule would alter the structure of the Fc portion in a way which would cause it to activate the immune effector systems. An alternate mechanism for triggering requires that two or more immunoglobulin molecules be brought in close proximity, as occurs when antibodies of the IgG class interact with multivalent antigens. In cases where the receptors for immunoglobulin are multivalent (e.g., Clq binds greater than 10 IgG molecules (Schumaker et al., 1976) and some cells bind 10^5 to 10^6 immunoglobulin molecules (Unkeless and Eisen, 1975; Kulczycki and Metzger, 1974)), the overall binding constant for a complex containing *n* immunoglobulin molecules would approximate the binding constant for one molecule to the *n*th power. In this way, the affinity of antibody for these receptors could be increased tremendously, even in relatively small complexes.

In order to study the relationship between antigenic recognition and effector system triggering, it would be desirable to

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[1] Abbreviations used: IgG, immunoglobulin G; Z, benzyloxycarbonyl; NHS, *N*-hydroxysuccinimide; DCC, dicyclohexylcarbodiimide; FDNB, 1-fluoro-2,4-dinitrobenzene; Dnp, 2,4-dinitrophenyl; Tnp, 2,4,6-trinitrophenyl; BADL, α-bromoacetyl-ε-Dnp-lysine; Me_2F, dimethylformamide; Me_2SO, dimethyl sulfoxide; $(BADL\text{-}Pro)_2$-EDA, bis(α-bromoacetyl-ε-Dnp-Lys-Pro)ethylenediamine.