CHARACTERIZATION OF THE NATIVE AND DENATURED FORMS OF $tRNA^{Trp}$ BY REACTION WITH KETHOXAL*

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Received 8 August 1973
Revised version received 13 February 1974

1. Introduction

Certain tRNAs have biologically inactive denatured conformations which are metastable at low temperatures in the presence of Mg^{2+} [1]. The existence of such forms was first noted by Lindahl et al. [2] for yeast $tRNA^{Leu}$. Since then, similar denatured forms have been discovered for *E. coli* $tRNA^{Trp}$ [3] and yeast $tRNA^{Ser}$ and $tRNA^{Phe}$ [4]. Denaturation of these tRNAs occurs upon heating above a transition temperature specific to the tRNA in the presence of EDTA. Renaturation is also accomplished by heating but in the presence of Mg^{2+}.

Upon denaturation, changes in secondary and tertiary structure occur. Using kethoxal (β-ethoxy-α-ketobutyraldehyde) as probe, we have investigated the conformations of both the denatured (D) and native (N) forms of $tRNA^{Trp}$ from *E. coli*. This reagent reacts specifically with guanines in exposed regions of tRNA (Litt [5], Litt and Greenspan [6]). We have ascertained differences in reactivity of the two forms of the tRNA and have shown that sites of reaction with kethoxal in the reactive D form are in the 3'-side of the anticodon stem, the D stem and the D loop. It appears that denaturation of this tRNA is accompanied by changes in hydrogen bonding in the anticodon and D stems.

2. Materials and methods

For the preparation of $[^{32}P]tRNA^{Trp}$, *E. coli* strain MRE 600 was grown in the medium of Garen and Levinthal [7], with amino acids substituted for Bacto-peptone. After addition of carrier (1–2 A_{260} units crude *E. coli* tRNA/ml culture), total tRNA was isolated by phenol extraction and DEAE-cellulose chromatography [8]. The tRNA was fractionated on a 0.4 × 12 cm BD-cellulose column (Gillam et al. [9]. A solution of 0.8 M NaCl in Buffer A (10 mM $MgCl_2$, 2 mM dithiothreitol, and 10 mM Na acetate, pH 4.5) was used to dissolve the tRNA, equilibrate the column, and elute about 80% of the radioactivity applied. Partly purified $tRNA^{Trp}$ was then eluted with Buffer A containing 1 M NaCl and 15% ethanol, ethanol precipitated, renatured, aminoacylated with $[^3H]$tryptophan (2–3 Ci/mmol, Amersham Searle) [3], and phenoxyacetylated [10]. The derivatized tRNA, dissolved in Buffer A plus 0.8 M NaCl, was rechromatographed on BD-cellulose. The bulk of the underivatized tRNA was removed by elution with this buffer, then with Buffer A plus 1.25 M NaCl and 12% ethanol. A 200 ml linear gradient was then applied from 12 to 25% ethanol in Buffer A containing 1.25 M NaCl. From those fractions with the highest $^3H/^{32}P$ ratio, $tRNA^{Trp}$ was ethanol preci-

* Supported by Grant CA-13173 from the National Institutes of Health and a grant from the American Cancer Society, Oregon Division.

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*** Career Development Awardee of the National Institute of General Medical Sciences.

Table 1
Molar ratios[a] of T1-ribonuclease digestion products of kethoxalated and control $tRNA^{Trp}$

Spot no.[f]	Sequence[e]	Found[b]	Control calculated	Ref. [8]	Kethoxalated	
					N form found[d]	D form found[c]
T1	G	12.1 ± 1.6	9	8.9	15.0	12.2 ± 1.5
T2	AG	3.4 ± 1.0	3	3.1	5.3	3.8 ± 0.4
T3	CG	1.5 ± 0.3	1	1.1	2.1	2.1 ± 0.4
T4	CACCG	1.2 ± 0.5	1	0.9	1.0	0.9 ± 0.1
T5	DAG	1.3 ± 0.2	1	0.9	1.1	1.3 ± 0.2
T6	U*AG	0.4 ± 0.1	1	0.5	0.4	0.3 ± 0.1
T7	pAG	0.8 ± 0.1	1	0.8	1.0	1.3 ± 0.2
T8	CCCCUG	(1.0)	1	1.0	(1.0)	(1.0)
T9	TΨCG	1.5 ± 0.2	1	1.1	1.5	1.7 ± 0.1
T10	Um^7GUUG	1.1 ± 0.2	1	1.0	0.9	0.6 ± 0.1
T11	UUCAADDG[g]	0.8 ± 0.1	1	0.5	0.9	1.1 ± 0.2
T12	UCUCUCCG	1.0 ± 0.1	1	1.0	1.1	1.2 ± 0.0
T13	$UCmUCCAA^*AACCG$	0.8 ± 0.1	1	0.9	1.2	0.2 ± 0.1
T15	$[UCmUCCAA^*AACCG^KG]$					0.6 ± 0.1
T16	$[G^KUm^7GUUG]$					0.3 ± 0.1

Kethoxalations were for 90 min as detailed under Methods. [a]Molar ratios were calculated from the radioactivity in each spot and are expressed relative to T8, taken as 1.0. Values given are averages ± 1/2 the range. Single determinations were averaged from each of [b]three preparations, [c]two preparations, and [d]one preparation. [e]Sequences were assumed from spot positions on the radioautograms, with compositions of all new spots and selected control spots confirmed by analyses of alkaline hydrolysates. [f]Spots were numbered as in ref. [8]. T14, a photo-oxidation product of T6 and T11 was not found on our autoradiographs. [g]The sequence shown is for T11 from control digests. T11 from the kethoxalated D form had a base composition of 20% G, 22% A, 12% C, 28% U and 18% D. U* is a modified uridine, probably 4-thiouridine; A* is a modified adenosine, probably 2-methylthio-6-isopentyl adenosine [8]; G^K is kethoxalated guanosine.

pitated, millipore filtered [11], and eluted and stripped of the derivatized amino acid with 1 M Tris–Cl, pH 8.0, held at 37°C for 30 min [12]. The $tRNA^{Trp}$ was judged about 68% pure as the molar ratio of TΨCG to CCCCUG (assumed unique to $tRNA^{Trp}$) is 1.5, rather than 1.0 as predicted from the structure (table 1) [8].

Kethoxalated or control [^{32}P]$tRNA^{Trp}$ was digested in the presence of borate by either T1-RNase (Sankyo) or pancreatic RNase (Rnase A, Sigma) [6]. The resulting oligonucleotides were resolved by two-dimensional high voltage electrophoresis, autoradiographed, counted, eluted, and alkaline hydrolyzed (Sanger et al. [13], with minor modifications of N. Pace, personal comm.). Base compositions were obtained by two dimensional thin layer chromatography [14].

The $tRNA^{Trp}$ was denatured by heating at 50–60°C for 15 min in 0.1 M Na cacodylate, pH 7.0, 1 mM EDTA, and renatured by so heating in the presence of 20 mM $MgCl_2$ [3]. Reaction of tRNA with kethoxal, borate stabilization of kethoxal adducts during RNase digestions and preparation of crude aminoacyl tRNA synthetases were described previously [6].

3. Results and discussion

3.1. *Kinetics of reaction of [3H] kethoxal with $tRNA^{Trp}$*

Fig. 1 shows the time course of reaction of $tRNA^{Trp}$ with kethoxal. After 90 min, about 2.0 moles of kethoxal have reacted per mole of the D form. A subsequent slower reaction follows which is independent of the presence of Mg^{++}. Under the same conditions there is little kethoxalation of the N form.

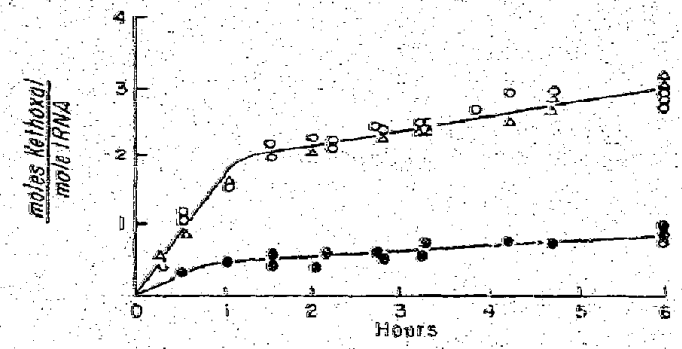

Fig. 1. Kinetics of reaction of [^{3}H]kethoxal with $tRNA^{Trp}$. Reaction mixtures contained, per milliliter final volume, 19 A_{260} units tRNA, 100 μmoles Na cacodylate (pH 7.0), 15 μmoles of [^{3}H]kethoxal, and unless otherwise noted, 10 μmoles of $MgCl_2$. Each point represents the average of two determinations. Solid circles, renatured $tRNA^{Trp}$; open circles, denatured $tRNA^{Trp}$; triangles, denatured $tRNA^{Trp}$ in the absence of $MgCl_2$.

3.2. *Location of kethoxalated residues*

Since a kethoxal–guanine complex protects the molecule from hydrolysis by T1-RNase adjacent to the site of modification, the reaction of kethoxal with tRNA should cause fingerprints of T1-RNase digests to show losses in spots present on fingerprints of untreated controls and a corresponding appearance of new spots which contain at least two guanines per mole of oligonucleotide (see below for base analyses).

Fig. 2A and 2B show T1-RNase fingerprints of untreated and kethoxalated D forms. Table 1 lists the molar ratios of the oligonucleotides obtained from these fingerprints. In the fingerprint of the kethoxalated D form, spot 13 is markedly reduced and four new spots, T15, T16, T17, and T18 are found. T15 is the major new spot.

Kethoxalation does not affect sites of hydrolysis by pancreatic RNase; however, kethoxalation of a residue

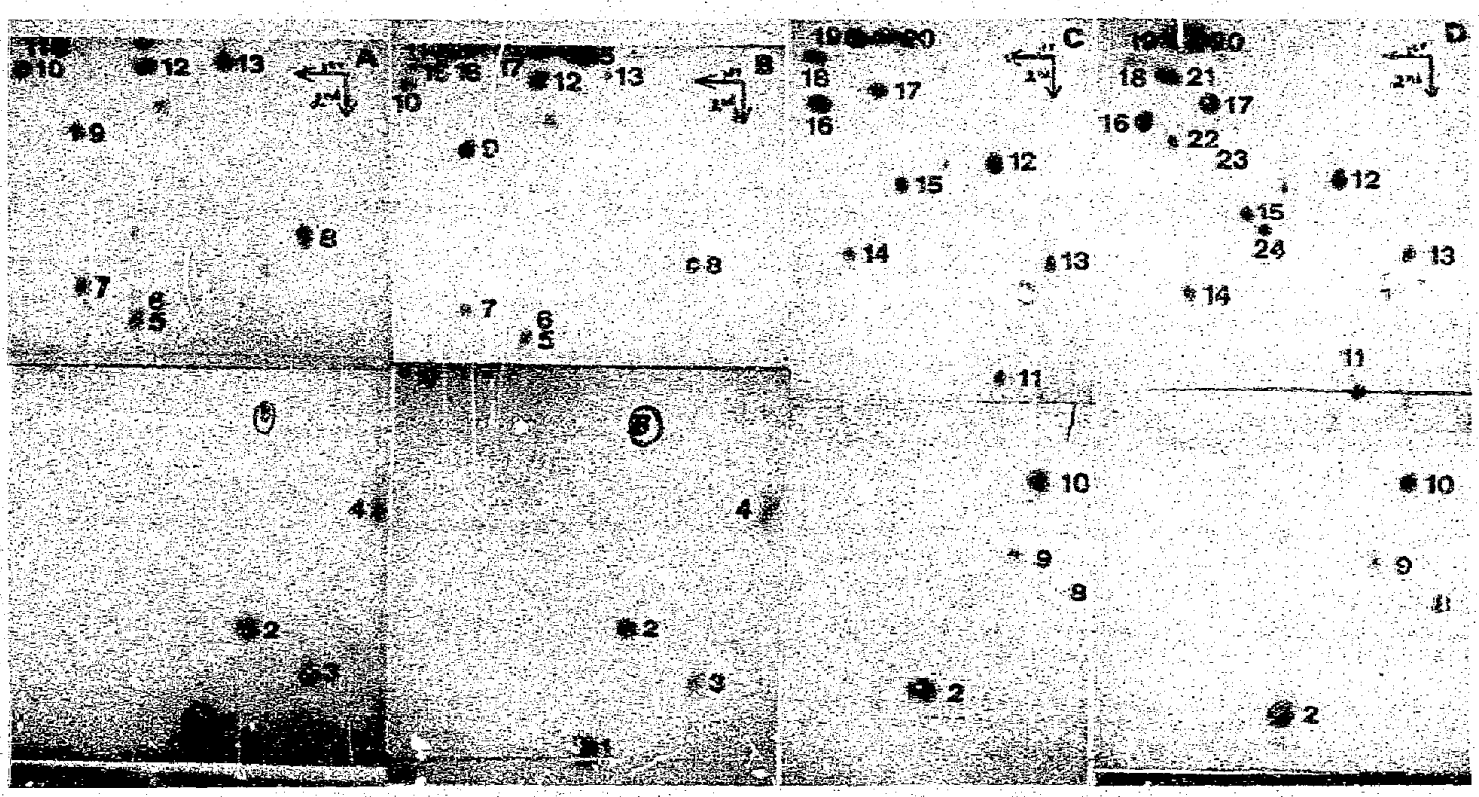

Fig. 2. Two dimensional electrophoretic patterns of T1- and pancreatic-RNase digestion products of $tRNA^{Trp}$ as described in the text. B is the blue marker. Spots are numbered as in ref. [8]. T1-RNase digests: A, unkethoxalated control, B, 90 min kethoxalated D form. Pancreatic-RNase digests: C, unkethoxalated control, D, 90 min kethoxalated D form.

Table 2
Molar ratios[a] of pancreatic ribonuclease digestion products of kethoxalated and control RNA^{Trp}

Spot no[f]	Sequence[e]	Found[b]	Control calculated	Ref. [8]	Kethoxalated	
					N form found[d]	D form found[c]
P8	m^7GU	0.5 ± 0.1	1	0.5	0.3	0.5 ± 0.1
P9	CmU	0.8 ± 0.2	1	0.8	0.7	0.9 ± 0.1
P10	GC	2.4 ± 0.4	2	2.3	1.6	1.9 ± 0.1
P11	AAD	0.8 ± 0.1	1	0.7	0.6	0.9 ± 0.2
P12	AGAGC	0.6 ± 0.1	1	1.0	0.8	0.7 ± 0.2
P13	AA*AAG	0.6 ± 0.2	1	0.9	0.5	0.7 ± 0.1
P14	GU	0.6 ± 0.2	1	0.5	0.5	0.9 ± 0.3
P15	AGU	0.9 ± 0.1	1	1.0	0.8	0.6 ± 0.2
P16	GGD, GGU	1.9 ± 0.1	2	1.8	1.6	1.2 ± 0.1
P17	GAGU	(1.0)	1	(1.0)	(1.0)	(1.0)
P18	GGGU	0.8 ± 0.1	1	0.8	0.9	0.3 ± 0.1
P19	GGGAGT	0.5 ± 0.2	1	0.8	0.8	0.6 ± 0.2
P20	pAGCGGC	0.4 ± 0.2	1	0.5	0.6	0.4 ± 0.2
P21	$(G^KGG)U$	–	–	–	–	0.6 ± 0.3
P22	$(G^KG)D$	–	–	–	–	0.4 ± 0.2
P23	G^KG^KD	–	–	–	–	0.1 ± 0.1
P24	$(AG^K)U$	0, trace[g]	–	–	–	0.5, 0.8[g]

Kethoxalations were performed as described in table 1. [a]Data were obtained as described in table 1 and are expressed relative to P17 as 1.0. b, d, e and f are as defined in table 1. [g]Data for individual runs are presented. The first and second values in each case are from the same tRNA preparations.

in a pancreatic oligonucleotide might be expected to shift its position on a fingerprint. Indeed, three new spots, P21, P22, P23 are seen on all fingerprints of the 90 min kethoxalated D form and P24, which was present as a trace spot on some control fingerprints, became prominent after kethoxalation (see fig. 2C, 2D). P21 was present in greater molar amounts than P22 (see table 2), and both were present in larger relative amounts than P23, a consistent but minor component. P18, P16 and P15 were reduced in these fingerprints.

Kethoxalation of the D form for 45 min gives patterns similar to those described above. No changes in the fingerprint pattern (not shown) or the molar ratios of oligonucleotides (tables 1 and 2) were found for the kethoxalated N form digested by either T1- or pancreatic RNase.

Because of the alkali lability of the kethoxal–guanine adduct [15], new spots on the pancreatic RNase fingerprints should give the same base analysis as those from which they are derived. P18 and P21 both analyse as GGGU, but only P18 appears on control fingerprints. Further, the amount of P21 corresponds to the loss of P18. Hence, P21 is inferred to be a kethoxalated derivative of GGGU. Similarly, the presence of two new spots, P22 and P23, both of which analyse as GGD, may be ascribed to the formation of mono- and di-kethoxalated derivatives of GGD. As predicted, the amount of P16 + P22 + P23 in the kethoxalated D form equals the amount of P16 in the control. By analogous reasoning, P24 is inferred to be $(AG^K)U$. From this data positions 10, 18, 19, and 42 are identified as possible sites of preferential kethoxalation in the D form.

Base analyses of oligonucleotides derived from T1-RNase digestions further define the kethoxalated residues. T15, the major new spot on the fingerprints of kethoxalated D form, appears to contain residues 31–42 as does control spot T13, plus an extra G. It could have arisen from T13 by kethoxalation of G30

or G42. Only the latter possibility is consistent with our analysis of the pancreatic RNase digests. The minor new spot T16 is seen similarly to have arisen from kethoxalation of G44. As predicted, the amount of T13 in the control equals the amount of T13+T15 present in the kethoxalated D form and T10 in the former equals T10+T16 in the latter. T17 apparently arose from kethoxalation of a contaminant as its base analysis was inconsistent with its having arisen from $tRNA^{Trp}$.

The pancreatic RNase data implicate G18 and G19 as kethoxalation sites. The undiminished yield of T5 in T1 digests of the kethoxalated D form suggest that G18 rather than G19 is the main site. The pancreatic RNase data also indicate that G10 is a kethoxalation site. This implies that fragments containing residues 8–18 and/or 8–19 should be present in T1 digests of kethoxalated D form. Unfortunately, our T1 data, although consistent with this possibility, are inconclusive. Thus, in fingerprints of kethoxalated D form, T11 is broader than in control fingerprints and has a significantly higher G content (table 1, footnote g). It may be a mixture of unmodified T11 with a fragment arising by kethoxalation at G10 or G18. That one of these fragments might be present in T18 is suggested by the presence of dihydrouridine in this spot. However, the base analysis of T18 does not agree with that predicted for any of these fragments. Perhaps T18 is contaminated with kethoxalated derivative arising from impurities in our tRNA preparations.

Fingerprints of T1 digests of N form after 90 min. kethoxalation had no spots not present in controls. The small extent of kethoxalation of the N form (fig. 1) probably reflects partial modification at several sites, not one of which is sufficiently modified to give rise to a new spot visible on our fingerprints. This interpretation is supported (data not shown) by DEAE-cellulose column profiles of T1 digests of kethoxalated N form prepared with a 70% pure preparation of non-radioactive $tRNA^{Trp}$ and [^{3}H]kethoxal.

Our conclusions are summarized in the cloverleaf representation of the tRNA sequence (fig. 3). Thus, in the denatured form, G42, on the 3'-side of the anticodon stem, is the most reactive with kethoxal, while G10 and G18 are the next most reactive. Since less than one mole of kethoxal per mole of tRNA is incorporated at each of these sites, our data suggest the

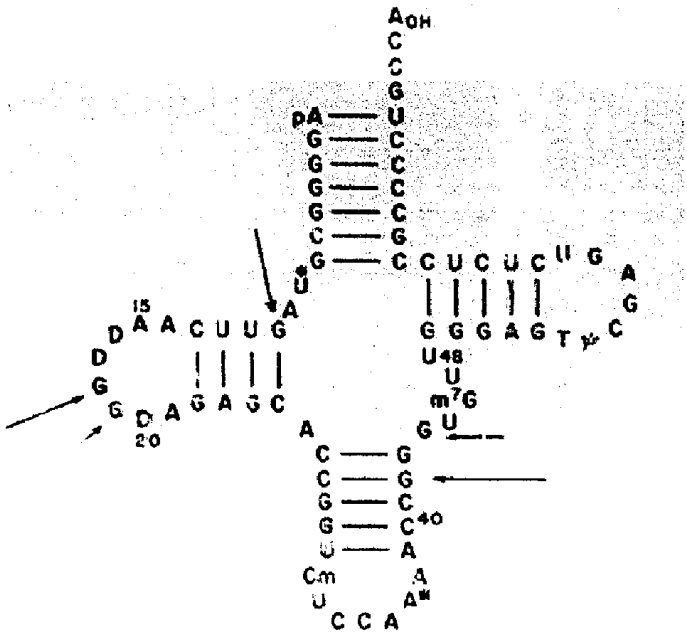

Fig. 3. Cloverleaf model of $tRNA^{Trp}$ from *E. coli* [8]. Arrows indicate positions of preferential kethoxalation of the denatured tRNA. The length of the arrow indicates relative reactivity of the site.

existence of several denatured states with different conformations.

Interestingly, yeast $tRNA^{Leu}$ also reacts readily with kethoxal only in the D state (Greenspan and Litt, unpublished observations). In this case the secondary slow reaction is accelerated by the absence of Mg^{2+}. Some native tRNAs do react readily with kethoxal. In fact, in yeast $tRNA^{Phe}$ and in *E. coli* $tRNA^{Val}$, position 20 in the D loop is accessible [6].

Other studies on conformational differences between D and N states using other chemical and physical probes for exposed regions have in all cases indicated that hydrogen bonds appear to be broken in the denaturation process [4, 16–20]. In no two denaturable tRNAs is the pattern of conformational change observed to be the same.

Acknowledgements

The authors wish to thank Charles Lytle for his excellent technical assistance, and Dr. Norman Pace for his hospitality to and expert training of C.M.G.

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