Loss of protection by nucleotides against proteolysis and thiol modification in the isolated α -subunit from F_1 ATPase of *Escherichia coli* mutant *uncA401*

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Binding of nucleotides to the high-affinity site of the isolated α subunit of normal *Escherichia coli* F_1 adenosine triphosphatase (ATPase) results in partial protection against digestion by trypsin [Senda, Kanazawa, Tsuchiya & Futai (1983) *Arch. Biochem. Biophys.* **220**, 398–440]. In contrast, the isolated α subunit from the defective ATPase of the *E. coli* *uncA401* mutant (strain AN120) is cleaved by trypsin to peptides of less than 8000 Da in the presence of ADP or ATP (2.5 μ M–110 mM). The nucleotide-dependent accessibility of thiol groups of the isolated α subunit was also studied. Two out of four thiol groups of the α subunit from normal ATPase are labelled by fluorescent maleimides or iodoacetates, but in the presence of ADP or ATP (0.14–1.2 mM), reaction of thiol groups with these labels is almost absent. Mutant α subunit, however, is labelled by these reagents at all four thiol groups in the presence or absence of ADP or ATP (1 mM). These results suggest that the mutation in the ATPase of strain AN120 leads either to the loss of the high-affinity nucleotide-binding site or affects transmission of allosteric changes that occur on binding of nucleotide to the isolated α subunit.

The peripheral portion (F_1) of the *E. coli* membrane ATPase complex contains five subunits designated α – ϵ with a likely stoichiometry of $\alpha_3\beta_3\gamma\delta\epsilon$ (Stan-Lotter & Bragg, 1984; for recent reviews see Futai & Kanazawa, 1983; Senior & Wise, 1983; Bragg, 1984). The defective ATPase from the *E. coli* mutant AN120 (*uncA401*) has less than 1% of the normal activity, although the enzyme complex is of apparently normal size, subunit composition and membrane-binding properties (Bragg & Hou, 1977; Senior *et al.*, 1979). The mutation has been located in the *uncA* gene, which codes for the α subunit (Kanazawa *et al.*, 1978; Dunn, 1978).

The *E. coli* ATPase contains three tightly-bound nucleotides which apparently do not participate in

catalysis (Bragg & Hou, 1977; Maeda *et al.*, 1977). The isolated α subunit from the normal ATPase binds ADP or ATP with high affinity (Dunn & Futai, 1980). It has been suggested that the α subunit might carry the tightly-bound nucleotides found in the ATPase (Dunn & Heppel, 1981). In addition, there are several low-affinity binding sites for nucleotides in the *E. coli* ATPase (Bragg *et al.*, 1981, 1982). These might be involved in catalysis and/or regulation. Three of these 'loose binding sites' are located in the α subunits (Bragg *et al.*, 1981; Verheijen *et al.*, 1980). Using the 2',3'-dialdehyde derivatives of ADP or ATP (oADP and oATP), we found previously that an oADP-binding site was absent from the ATPase from the mutant AN120 (Bragg & Hou, 1980a). A similar result was obtained for an *uncA* mutant by Verheijen *et al.* (1980) using 8-azido-ATP.

In contrast with the above, essentially the same number of nucleotide-binding sites can be found in *uncA* mutant ATPase when direct binding of ATP (Bragg, 1984), 5'-adenylyl imidodiphosphate (Wise *et al.*, 1984) or 2'-(5-dimethylaminonaphthalene-1-sulphonyl)amino-2'-deoxyATP (Kanazawa *et al.*, 1984) is measured. However, there appears to be

Abbreviations used: F_1 ATPase, (Ca^{2+} + Mg^{2+})-activated adenosine triphosphatase (EC 3.6.1.3); MIANS, 2-[(4'-maleimidyl)anilino]naphthalene-6-sulphonic acid; IAANS, 2-[4'-iodoacetamido]anilino]naphthalene-6-sulphonic acid; IAEDANS, 5-[2-(iodoacetyl)aminoethyl]aminonaphthalene-1-sulphonic acid; SDS, sodium dodecyl sulphate; oATP and oADP, 2',3'-dialdehyde derivatives of adenosine 5'-tri- and di-phosphates.

an alteration in the affinity of at least one of the nucleotide-binding sites in the *uncA* mutant ATPase (Bragg *et al.*, 1982; Bragg, 1984; Wise *et al.*, 1984).

Binding of nucleotides to the isolated α subunit from normal ATPase has been demonstrated with labelled nucleotides (Dunn, 1980), by measurements of physical properties (Dunn, 1980; Paradies, 1980, 1981) and resistance to trypsin treatment (Senda *et al.*, 1983). Using non-denaturing gel electrophoresis (Dunn, 1980), we obtained evidence for the lack of ATP binding by the α subunit of *uncA401* mutant ATPase (Bragg *et al.*, 1982). However, dissociation of nucleotide from the protein during electrophoresis could not be ruled out entirely, particularly if the affinity was low. In view of the uncertainty regarding the nature of the nucleotide-binding site of the α subunit in the *uncA401* ATPase, we have investigated the binding of ADP and ATP to the isolated α subunit using methods where a high nucleotide concentration could be maintained throughout the experiment. The remarkable effect of partial resistance to trypsin treatment of normal α subunit in the presence of nucleotides, discovered by Senda *et al.* (1983), was suitable for this purpose. Changes in the accessibility of thiol groups to fluorescent maleimides in the presence and absence of nucleotides were also examined (Futai *et al.*, 1982). We found maleimide-derived reagents not to be specific for thiol groups in the α subunit, and we eliminated this shortcoming by using fluorescent iodoacetates. With these methods, we could not detect a high-affinity nucleotide-binding site in the isolated α -subunit from the *uncA401* mutant ATPase.

Materials and methods

Materials

The following materials were supplied by the companies indicated. Bio-Rad: acrylamide, bis-acrylamide, SDS, urea, Bio-Gel P-10, Bio-Rad protein assay reagent; Sigma Chemical Co.: ATP, iodoacetamide, iodoacetic acid, dithiothreitol, Coomassie Blue, trypsin (treated with diphenyl carbamoyl chloride), soya bean trypsin inhibitor, bovine serum albumin; Pharmacia Fine Chemicals: ADP, ampholytes; Molecular Probes Inc.: MIANS (sodium salt), 5-maleimidylsalicylic acid, IAANS (sodium salt), IAEDANS.

Preparation of ATPase

The ATPase of the normal strain (*E. coli* ML308-225) and the *uncA401* mutant (*E. coli* AN120) were purified as described previously (Bragg & Hou, 1977, 1980b) except that separation of the enzymes on a sucrose gradient was omitted.

E. coli AN120 was generously given by Professor Frank Gibson, Canberra, Australia.

Preparation of α subunit of ATPase

The α subunits of the ATPases from strains ML308-225 and AN120 were prepared as described previously (Dunn & Futai, 1980; Bragg *et al.*, 1982).

Treatment of α subunit with trypsin

The method of Senda *et al.* (1983) was used with minor modifications. Isolated α subunit (0.45 mg/ml) from normal ATPase was incubated at 23°C in 50 mM-Tris/HCl (pH 8.0)/0.1 mM-EDTA/0.3 mM-2-mercaptoethanol/0.1 M-NaCl with 23 μ g of diphenyl carbamoyl chloride-treated trypsin/ml. Proteolysis was terminated after the times indicated by adding soya bean trypsin inhibitor (2-fold excess over trypsin). α Subunit from *uncA401* mutant ATPase (0.24 mg/ml) was treated similarly with trypsin/protein ratios of 1:20–1:100. When nucleotides were added to the α -subunit (as indicated in the Results section), the mixture was left for 15 min at 23°C before adding trypsin.

Labelling of thiol groups of α subunit

Labelling with fluorescent reagents was carried out under dim illumination. Stock solutions of fluorescent reagents were stored at 4°C in the dark for not longer than 24 h.

Isolated α subunit (0.83 mg/ml) from normal ATPase was incubated with 2.8 mM-MIANS in 50 mM-Tris/HCl (pH 8.0)/0.1 mM-EDTA/0.1 mM-dithiothreitol/0.1 M-NaCl at 23°C in the dark for 20 min. A stock solution of MIANS in dimethylformamide at a concentration of 10 mg/ml was used. Free MIANS was removed from the α subunit by desalting on Bio-Gel P-10 in 50 mM-NH₄HCO₃ adjusted to pH 8.35 with NH₄OH, using the centrifuged column method (Peneffsky, 1977). Isolated α -subunit (0.12 mg/ml) from *uncA401* mutant ATPase was treated similarly and, after acidification of the column effluent with dilute acetic acid, concentrated by lyophilization. Nucleotides were added to the α subunit (as indicated in the Results section) and were left for 15 min at 23°C before adding the labelling reagents. Labelling of α subunit from normal ATPase with 5-maleimidylsalicylic acid was performed as described for MIANS. The reagent was used at a final concentration of 0.5 mM; its stock solution was made in dilute NaOH at a concentration of 5 mg/ml. Labelling of α subunits with IAANS was carried out as described for MIANS except that the incubation time was 1.5 h; the final concentration of IAANS was 0.6 mM and its stock solution was made in dimethylformamide at a concentration of 5 mg/ml. The presence of fluorescent

labelled α subunits was detected under u.v. light immediately after gel electrophoresis before staining the gel.

Protein determination

Protein was determined by the method of Bradford (1976) with bovine serum albumin as a standard. For the α subunit, values were corrected by a factor of 1.2 (Dunn, 1980). Alternatively, a specific absorption coefficient $A_{1\text{cm}, 280}^{1\%}$ of 5.3 was used for the α subunit. This was based on the protein content determined by the Bradford assay.

Gel electrophoresis

SDS/polyacrylamide-gel electrophoresis was carried out according to Laemmli (1970). For iso-electric focusing gels the system of O'Farrell (1975) was used with the modifications described previously (Stan-Lotter & Bragg, 1984). Gels were stained with Coomassie Blue (Fairbanks *et al.*, 1971) or with silver (Merril *et al.*, 1983).

Results

Effect of ADP and ATP on the trypsin sensitivity of the α subunit from normal and *uncA401* mutant ATPase

The α subunit from the *E. coli* ATPase has an M_r of 55264 as calculated from the amino acid sequence (Gay & Walker, 1981; Kanazawa *et al.*, 1981). A total of 54 arginine and lysine residues are present, and trypsin cleaves the protein to small polypeptides of less than 8000 Da (Senda *et al.*, 1983). In the presence of ADP and ATP, however, the α subunit is cleaved by trypsin to two main polypeptides of 31000 and 25000 Da; with increased times of incubation the 31000-Da fragment is digested further to a 30000-Da polypeptide (Senda *et al.*, 1983).

The isolated α subunit from the normal ATPase was cleaved by trypsin in the presence of 16 μM-ATP or ADP to the two expected fragments of 30000–31000 and 25000 Da after 30 min of incubation (Fig. 1, lanes 2 and 3). When the isolated α subunit from *uncA401* mutant ATPase was treated with trypsin in the presence of 2.5 μM-ATP or ADP (Fig. 1, lanes 5 and 6) or in the absence of nucleotides (Fig. 1, lane 7) the protein was digested to peptides of less than 8000 Da. Increased concentrations of nucleotides (1.4 or 14 mM-ATP; 1.4, 14 or 110 mM-ADP) or the addition of Mg²⁺ (180 mM) in the presence of ADP (90 mM) during trypsin treatment of mutant α subunit resulted in essentially the same electrophoretic pattern as in Fig. 1, lanes 4–7. Likewise, decreased incubation times (20 min) or performing the treatment at pH 7.3 did not alter the pattern.

Fig. 1. Trypsin treatment of isolated α subunits from ATPases of normal and *uncA401* mutant strains of *E. coli*. α subunits from normal (135 μg) or *uncA* mutant (75 μg) ATPase were incubated with trypsin (7 μg for normal, 0.75 μg for mutant α subunit) for 30 min in the presence of nucleotides. The reaction was terminated with trypsin inhibitor (see the Materials and methods section). Samples were submitted to SDS/polyacrylamide-gel electrophoresis (Laemmli, 1970) on a 13% gel which was stained with Coomassie Blue. Lane 1, 6 μg of normal α subunit without trypsin treatment; lanes 2 and 3, normal α subunit treated with trypsin in the presence of 16 μM-ATP (lane 2) or ADP (lane 3); lane 4, 4.5 μg of mutant α subunit without trypsin treatment; lanes 5 and 6, mutant α subunit treated with trypsin in the presence of 2.5 μM-ATP (lane 5) or ADP (lane 6); lane 7, mutant α subunit treated with trypsin in the absence of nucleotides. The 30000 (30K), 31000 (31K) and 25000 (25K) Da polypeptides are indicated, as well as the position of trypsin inhibitor (ti).

These results indicate that nucleotides do not protect the isolated α subunit from the *uncA401* mutant ATPase against digestion by trypsin.

Accessibility of thiol groups of α subunits from normal and *uncA401* mutant ATPases in the presence and absence of ADP and ATP

We reported that the α subunit of normal ATPase possesses four thiol groups, which are completely labelled with SH-specific reagents when the enzyme is denatured in urea (Stan-Lotter & Bragg, 1984). The number of thiol groups can be most readily detected by the method of Creighton (1980), which is based on the following considerations. By reacting cysteine-containing denatured and reduced polypeptides with iodoacetate, acidic carboxymethyl groups are introduced, while reaction with the neutral iodoacetamide causes no

charge differences. By using various ratios of iodoacetamide to iodoacetate a spectrum of polypeptide molecules is generated having 0 to n acidic carboxymethyl groups, where n is the number of cysteine residues/molecule of polypeptide. When these species are separated by polyacrylamide-gel electrophoresis on the basis of charge, $n+1$ species will be visible after staining for protein. Thus, an integral number of cysteine groups can be determined by counting the bands. Using iodoacetate alone, complete modification of the protein can be observed with the generation of one single species possessing n additional negative charges and migrating accordingly in a suitable separating system.

The α subunit of the *E. coli* ATPase may show some charge heterogeneity, with generally one predominant species and up to two minor species present (Fig. 2, lanes 1 and 4). This may lead to the formation of additional bands, since each species is modified independently. However, this does not result in any ambiguity as to the extent of modification.

The ATPase from the *uncA401* mutant was modified with mixtures of iodoacetamide and iodoacetate and separated on an isoelectric focusing gel (Fig. 2, lane 3). Five species of α subunit were obtained, indicating the introduction of 0–4

acidic carboxymethyl groups and thus the presence of four thiol groups per molecule of mutant α subunit. This result is similar as that given by the normal ATPase (Fig. 2, lane 2).

The isolated α subunit from normal ATPase, which is reconstitutively active, is not appreciably labelled by the SH-specific reagents mentioned above. However, the α subunit reacts with several fluorescent maleimides and iodoacetates carrying negatively charged groups. This allows the identification of reacted α species on isoelectric focusing gels in a similar manner as described above.

Labelling of the isolated α subunit from normal ATPase with MIANS in the absence or presence of 0.14 mM-ATP is shown in Fig. 2. Following modification with MIANS in the absence of ATP (Fig. 2, lane 5), little of the original α species (Fig. 2, lane 4) can be detected. The new species with the lowest mobility has acquired two more charges than found in the original untreated species. Numerous other species with increasing mobility are present, indicating the introduction of numerous negative charges. Since these species extend far below the position '4' in Fig. 2, which designates the position of the α subunit with four additional negative charges, it can be concluded that other groups besides the thiol groups of the α subunit had been modified. These are presumably

Fig. 2. Labelling of α subunit from normal ATPase with MIANS

Isolated α subunit from normal ATPase was labelled with MIANS as described in the Materials and methods section in the presence or absence of ATP. After removing excess reagent, samples were denatured in urea and remaining free thiol groups were reacted with iodoacetate. Samples were run on isoelectric focusing gels with the system of O'Farrell (1975). Gels were stained with Coomassie Blue. As markers, α subunits with 0–4 additional negative charges are shown. These were obtained after reacting thiol groups of urea-denatured ATPase with mixtures of iodoacetamide and iodoacetate (Creighton, 1980). Lane 1, untreated normal ATPase (only the α subunit of the ATPase is shown in lanes 1–3); lanes 2 and 3, ATPase treated according to Creighton (1980) from normal (lane 2) or *uncA401* mutant (lane 3) strains; lane 4, untreated α subunit from normal ATPase; lanes 5 and 7, α subunit labelled with MIANS in the absence (lane 5) or presence (lane 7) of 0.14 mM-ATP; lanes 6 and 8, same samples as in lanes 5 and 7, but after reaction with iodoacetate. The positions of the α subunits with 0–4 additional negative charges are indicated.

amino groups. In order to determine which groups are modified, the MIANS-treated sample was reacted subsequently with iodoacetate in 8M-urea. This resulted in the introduction of two further negative charges into the slowest moving species (Fig. 2, lane 6). Iodoacetate is strictly specific for thiol groups; thus we conclude that MIANS originally modified only two of the four thiol groups in the α subunit. If only amino groups had been modified by MIANS in Fig. 2, lane 5, the subsequent reaction with iodoacetate in 8M-urea should have introduced four negative charges. This would have resulted in a total of six additional negative charges for the slowest-moving species of modified α subunit.

In the presence of ATP a large portion of the α subunit did not react with MIANS (Fig. 2, lane 7). This suggested that modification of the two thiol groups detected previously did not occur. This was verified by reaction of the MIANS-treated sample with iodoacetate (Fig. 2, lane 8), where the slowest-moving α subunit acquired four additional negative charges. ADP (0.1 mM) had essentially the same effect as ATP in protecting the thiol groups

of normal α subunit from labelling with MIANS (not shown).

Labelling of the isolated α subunit from *uncA401* ATPase with MIANS in the absence or presence of 1 mM-ADP or ATP is shown in Fig. 3, lanes 2–4. In each case, at last four negative charges were introduced into the α subunit, indicating that all four thiol groups had been labelled by MIANS and that no protection was provided by the nucleotides. As before, amino groups appear to have been modified by MIANS with the consequent presence of numerous charged α-species.

The reaction of maleimides with amino groups under certain conditions is known (Smyth *et al.*, 1960; Brewer & Riehm, 1967). Reaction of MIANS with an amino group leads to a net incorporation of two negative charges into the protein, and this was frequently observed as an increased intensity of every second band of MIANS-labelled α subunit (e.g. Fig. 2, lane 7). Attempts to minimize this reaction by using short incubation times (5 min), different concentrations of the reagent (0.2–1.4 mM) or a different pH (7.0) were unsuccessful. Another maleimide-derived fluorescent reagent, 5-maleimidylsalicylic acid, reacted similarly with both thiol and amino groups of the α subunit.

Several iodoacetate-derived fluorescent reagents were found to be specific for thiol groups. IAANS and IAEDANS labelled urea-denatured α subunit at four thiol groups only, as shown evidenced by isoelectric focusing (results not shown). Native α subunit from normal ATPase was reacted with IAANS. In the absence of nucleotides, four thiol groups were labelled, two of them completely (Fig. 4, lane 2). In the presence of approx. 1 mM-ADP or ATP, a major portion of α subunit migrated at the same position as the untreated material, that is it remained unlabelled (Fig. 4, lanes 3 and 4). In contrast, the α subunit from the *uncA401* ATPase was labelled by IAANS in the absence or presence of 1 mM-ADP or ATP at four thiol groups (Fig. 4, lanes 5–7).

Fig. 3. Labelling of α subunit from *uncA401* mutant ATPase with MIANS

For details see the legend to Fig. 2. The isoelectric focusing gel was stained with silver (Merril *et al.*, 1983) after electrophoresis. Lane 1, untreated α subunit from mutant ATPase; lanes 2–4, α subunit from mutant ATPase labelled with MIANS in the absence (lane 2) or presence of 1 mM-ADP (lane 3) or 1 mM-ATP (lane 4). The positions of α subunits with 0 and 4 additional negative charges are indicated.

Discussion

The binding of nucleotides to the *E. coli* ATPase has been studied by using various ADP and ATP analogues (Bragg & Hou, 1980a, Bragg *et al.*, 1981, 1982; Bragg, 1984; Lunardi *et al.*, 1981; Wise *et al.*, 1981, 1984; Kanazawa *et al.*, 1984). These reports differ somewhat regarding the total number of bound nucleotides and the absence or presence of nucleotide-binding sites in *unc* mutants. This might be due to the different methods employed. When purified subunits of the bacterial ATPase became available, direct binding of ADP or ATP could be measured, for example, to the α subunit

Fig. 4. Labelling of α subunit from normal and *uncA401* mutant ATPase with IAANS

For details see the legend to Fig. 2. Labelling of α subunits with IAANS was carried out as described in the Materials and methods section. Lane 1, untreated α subunit from normal ATPase labelled with IAANS in the absence (lane 2) or presence of 1.2 mM-ADP (lane 3) or 1.1 mM-ATP (lane 4). Lanes 5–7, α subunit from mutant ATPase labelled with IAANS in the absence (lane 5) or presence of 1.1 mM-ADP (lane 6) or 1.0 mM-ATP (lane 7).

from the ATPase of *E. coli* (Dunn, 1980) or the thermophilic bacterium PS3 (Ohta *et al.*, 1980), or, more recently, to the β subunit from the *Rhodospirillum rubrum* ATPase (Gromet-Elhanan & Khananshvili, 1984).

Two types of experiments were used in the present study to investigate the binding of nucleotides to the isolated α subunit from the defective *uncA401* ATPase. In the α subunit from normal *E. coli* ATPase, the binding of ATP can be detected by the resistance of the α subunit–nucleotide complex to trypsin digestion (Senda *et al.*, 1983). This effect can be demonstrated with concentrations as low as 0.1 μ M-ATP, or about equimolar amounts of nucleotide and α subunit. This reflects the high affinity of the α subunit for ADP or ATP (K_D of 0.9 μ M or 0.1 μ M, respectively; Dunn & Futai, 1980). We found that the isolated α subunit from the *uncA401* mutant ATPase showed no resistance to trypsin digestion in the presence of nucleotides using nucleotide concentrations of up to 110 mM, which equals a 10^5 -fold molar excess over the α subunit.

A low-affinity binding site for ATP in the isolated β subunit of the *Rhodospirillum rubrum* ATPase has recently been demonstrated (Gromet-Elhanan & Khananshvili, 1984), with a nucleotide concentration of 1 mM. On the basis of the above data even a low-affinity nucleotide binding site is unlikely to exist in the isolated α subunit from the *uncA401* ATPase.

The accessibility of thiol groups of the α subunit of the normal ATPase to modification by SH-

specific reagents is also changed in the presence of nucleotides. Using *N*-(1-anilinoanthryl-4)-maleimide, Futai *et al.* (1982) found that less of the label reacted with the α subunit when ATP was present. Our experiments with similar thiol-specific fluorescent reagents essentially confirm and extend this result for the α subunit of the normal ATPase. We have found that two out of the four thiol groups of isolated normal α subunit may be protected against modification by the presence of nucleotides.

The thiol groups of the α subunit from the *uncA401* mutant ATPase were not protected from labelling by the same thiol-specific fluorescent reagents when nucleotides in concentrations of up to 1 mM were present. This confirms our previous conclusion that a high-affinity nucleotide-binding site is absent from the isolated α subunit from the *uncA401* mutant ATPase. The loss of a high-affinity nucleotide-binding site in the isolated mutant α subunit is probably reflected in the decrease in the affinity of the holoenzyme for nucleotides observed in the binding experiments (Bragg *et al.*, 1982; Bragg, 1984; Wise *et al.*, 1984).

The mutation in the α subunit of the *uncA401* ATPase is believed to be a point mutation and has recently been localized between the amino acid residues 370 and 387 (Kanazawa *et al.*, 1984). The M_r and pI are the same as for the normal α subunit (Dunn, 1978; Kanazawa *et al.*, 1978; Senior *et al.*, 1979). The amino acids possibly involved in nucleotide-binding sites of the α and β subunits of the *E. coli* ATPase have recently been outlined by

Walker *et al.* (1982*a,b*) on the basis of sequence homologies with known nucleotide-binding proteins. Such a nucleotide 'pocket' might involve as many as 90 residues from various portions of the polypeptide chain (Senior & Wise, 1983). It is difficult to imagine how a single mutation might completely abolish the nucleotide-binding capacity if the overall conformation of the protein remains the same. The nucleotide pocket might be disrupted, however, if the mutation leads to a total loss of the conformation which is essential for binding. Some evidence of differences in conformation between normal and mutant α subunits, such as chromatographic and electrophoretic behaviour (Bragg *et al.*, 1982) and the labelling of four thiol groups in mutant α subunits (compare Fig. 2, lane 5 and Fig. 3, lane 2; also Fig. 4, lanes 2 and 5), is at least consistent with this view. The possibility of a difference in conformation between normal and mutant α subunits (increased hydrophobicity in the mutant α subunit) has also been discussed by Kanazawa *et al.* (1984).

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