Biochimica et Biophysica Acta, 592 (1980) 519—527

BBA 47928

INTERACTION OF CYTOCHROME *c* WITH CYTOCHROME bc_1 COMPLEX OF THE MITOCHONDRIAL RESPIRATORY CHAIN

CLEMENS BROGER, MACIEJ J. NAŁĘCZ * and ANGELO AZZI

Medizinisch-chemisches Institut der Universität Bern, Bern (Switzerland)

(Received February 6th, 1980)

Key words; Cytochrome bc_1 complex; Cytochrome c; Photoaffinity labeling; Electron transfer; Respiratory chain

Summary

The binding of cytochrome *c* to the cytochrome bc_1 complex of bovine heart mitochondria was studied. Cytochrome *c* derivatives, arylazido-labeled at lysine 13 or lysine 22, were prepared and their properties as electron acceptors from the bc_1 complex were measured. Mixtures of bc_1 complex with cytochrome *c* derivatives were illuminated with ultraviolet light and afterwards subjected to polyacrylamide gel electrophoresis. The gels were analysed using dual-wavelength scanning at 280 minus 300 and 400 minus 430 nm. It was found that illumination with ultraviolet light in the presence of the lysine 13 derivative produced a diminution of the polypeptide of the bc_1 complex having molecular weight 30 000 (band IV) and formation of a new polypeptide composed of band IV and cytochrome *c*. Band IV was identified as cytochrome c_1, and it was concluded that this hemoprotein interacts with cytochrome *c* and contains its binding site in complex III of the mitochondrial respiratory chain. Illumination of the bc_1 complex in presence of the lysine 22 derivative did not produce changes of the polypeptide pattern.

Introduction

In recent years various techniques have been applied to the mitochondrial respiratory chain in order to elucidate the sequence of its electron transfer components. Spectroscopic techniques have been used, where applicable, to

* Present address: Nencki Institute of Experimental Biology, Department of Cellular Biochemistry, 02-093 Warsaw, Poland.

Abbreviations: NAP-13-cytochrome *c* and NAP-22-cytochrome *c* are derivatives of cytochrome *c* containing a nitroazidobenzene ring attached at lysine residue 13 and 22, respectively.

establish the sequence of reduction of the electron carriers and their standard redox potentials.

On the basis of kinetic evidence [1] it was found that cytochrome c_1 donates electrons to cytochrome *c*. Measurements of the standard redox potentials [2] of these cytochromes would indicate the same order. The mentioned sequence is also supported by results from studies with electron flow inhibitors [3]. Direct evidence, that cytochrome c_1 is the binding site for cytochrome *c*, however, is not yet available. Many other subunits may play a role in the bc_1 complex as binding site(s) for cytochrome *c* and the kinetic data would not reveal them if they had no spectroscopically detectable group. On the basis of its accessibility to dissociating agents and its high midpoint potential it is conceivable, for example, that the iron sulfur protein functions between cytochrome c_1 and *c* [4].

More recent experiments were devised to elucidate the structural aspects of the interaction between cytochrome *c* and cytochrome bc_1 complex. It was shown that the same lysine residues of cytochrome *c* became shielded when this protein formed a complex with cytochrome *c* reductase or with isolated cytochrome c_1 [5]. Also stability constants of these complexes, as well as of the complex formed between the cytochromes *c* and *b* were measured. The results indicated that cytochrome *c* combines with cytochrome *c* reductase [5,6] and isolated cytochrome c_1 [5,7] but not with isolated cytochrome *b* [5]. The interaction of other subunits of complex III with cytochrome *c* has not yet been studied. On the other hand, the region of the cytochrome *c* molecule interacting with the reductase has been defined [8,9].

In the investigation reported here, the binding of arylazido derivatives of cytochrome *c* to the purified bc_1 complex was studied. Upon illumination, the azido group was converted to a nitrene [10] able to react with the nearest C-H bond and consequently to cross-link the cytochrome *c* derivative to its binding site.

Similar experiments to those reported here were already performed to identify the subunit of cytochrome *c* oxidase interacting with cytochrome *c* [11].

Materials and Methods

Preparation of the bc_1 complex and measurement of its enzymatic activity

Mitochondria were prepared from bovine heart according to the method of Blair [12]. The bc_1 complex was isolated according to Hatefi and Rieske [13,14] and stored in small portions at −80°C. Enzymatic activity was measured according to the procedure of Wan and Folkers [15] using Coenzyme Q_1 as a substrate, which was reduced as described in Ref. 14.

Preparation of arylazido-cytochrome c derivatives

The arylazido labeling of cytochrome *c* was performed as described [11]. The cytochrome *c* was dissolved in 1.4 ml of 0.1 M sodium bicarbonate buffer, pH 8.5. 9 mg of 4-fluoro-3-nitrophenylazide in 100 µl of ethanol was added. The reaction was carried out in the dark with continuous stirring at 37°C. After 50 h, the reaction mixture was centrifuged in a bench centrifuge and the super-

natant passed through a Sephadex G-25 column equilibrated with 10 mM ammonium phosphate, pH 7.2. The labeled products were fractionated on a carboxymethyl-cellulose column (1 × 55 cm). Fractions of labeled cytochrome *c* were eluted over a period of 18 h with 25 mM ammonium acetate, pH 7.2 at a flow rate of 12 ml/h. The distribution of labeled cytochrome *c* showed two peaks, the former indicating a ratio of azido groups per cytochrome *c* molecule of more than two and the latter a ratio of two. Afterwards, the ammonium acetate concentration was increased linearly to 200 mM, the pH being maintained at 7.2, and fractions of singly-labeled and non-labeled cytochrome *c* were eluted. These fractions were dialysed against distilled water and concentrated by lyophilisation. The number of azido groups per cytochrome *c* molecule was determined from the ratio of absorbance at 480 nm to that at 408 nm and by electrophoresis on cellulose acetate strips in 50 mM Tris acetate, pH 7.2, at 200 V for 1 h. The location of the azido groups in the cytochrome *c* molecules was inferred [11] using pepsin digestion [16] followed by chromatography of the digests on thin layer plates in the solvent mixture 1-butanol/acetic acid/pyridine/water (10 : 15 : 3 : 12, v/v).

Interaction of cytochrome c with the bc_1 complex

A sample of isolated bc_1 complex was thawed and dialysed overnight at 4°C against 50 mM Tris-HCl, 0.4% sodium cholate, pH 8. The incubation mixture consisted of 200 μg of the bc_1 complex, 25 mM Tris-HCl, 0.2% sodium cholate and 12 μM cytochrome *c* derivative, pH 8, in a volume of 400 μl. After 15 min in the dark at 4°C, the mixtures were illuminated in glass cuvettes with a 100 watt ultraviolet lamp at 4°C for 1 h. After illumination, a small amount of sodium dithionite was added to the samples to reduce non-reacted azide and the mixture was passed through an Amberlite CG 50 column (5 × 20 mm) equilibrated with 50 mM ammonium acetate, pH 7.2, to remove most of the free cytochrome *c*. 50 μl of the eluate was mixed with the dissociating solution (see below) in equal proportions and applied to the polyacrylamide gels.

Electrophoresis

Electrophoresis was performed according to Weber and Osborn [17]. The protein was dispersed in the dissociating solution of 2% SDS, 10% mercaptoethanol, 50 mM sodium carbonate, 1 mM iodoacetic acid and 10% glycerol and incubated for 15 min on ice before electrophoresis. The electrophoresis apparatus was cooled with running water. The migration distance was about 7 cm. After electrophoresis the gels were placed in 12% trichloroacetic acid for 2 h and then washed overnight with 10% isopropanol, 7% acetic acid. Subsequently, they were put into quartz tubes and scanned with a gel-scanner attachment for an Aminco DW-2A dual wavelength spectrophotometer built in this laboratory [18]. Some gels were also stained with Coomassie blue as described by Weber and Osborn [17] and their absorbance at 600 nm was measured. Bovine serum albumin, ovalbumin, trypsin and cytochrome *c* were used as standards for molecular weight determination. In some experiments, gels were also stained with tetramethylbenzidine to detect heme. Directly after electrophoresis, the gels were placed into a warm saturated solution of tetramethylbenzidine in 10% isopropanol, 7% acetic acid and incubated for 15 min.

They were then placed into the scanning cuvette filled with 30% hydrogen peroxide. After 1 to 2 min the greenish-blue bands of heme-containing peptides were developed and the gels were immediately scanned at 540 nm.

Materials

Horse heart cytochrome *c* (type VI) was obtained from Sigma, 4-fluoro-3-nitrophenylazide from Molecular Probes, Roseville, Michigan, and Carboxymethyl cellulose from Whatman. Coenzyme Q_1 was a gift from Hoffmann-La Roche, Basel, Switzerland. All other reagents used were of the highest purity commercially available.

Results

Labeling of cytochrome c

Arylazido derivatives of cytochrome *c*, obtained from the reaction with 4-fluoro-3-nitro-phenylazide were separated by carboxymethyl cellulose chromatography into fractions of multi-, doubly-, singly- and non-labeled cytochrome *c*. In the fractions of singly-labeled derivatives, only cytochrome *c* labeled at either lysine 13 or lysine 22 should be present [11], and pepsin digestion and thin-layer chromatography of the derivatives [16] were in agreement with this. It was very difficult, however, to separate these two derivatives on the ion exchange column. However, although the net charge of the derivatives is exactly the same, the charge distribution is different in the lysine-13- and the lysine-22-labeled derivatives of cytochrome *c*, thus allowing their complete separation [11,19]. Although in our case it was impossible to obtain completely purified species, the ratio of lysine-13 to lysine-22 derivative became smaller the later the fraction was eluted from the carboxymethyl-cellulose column. The two fractions used in the present study are about 90% pure. They will be called NAP-13-cytochrome *c* and NAP-22-cytochrome *c*, indicating that the corresponding derivative was largely prevailing.

Enzymatic activity

The enzymatic activity of the purified bc_1 complex was measured with both arylazido cytochrome *c* derivatives as well as with native cytochrome *c*. In all cases the activity measured varied between 5 and 6 μmol of cytochrome *c* reduced per mg of complex III per min when 10 μM coenzyme Q_1 was used as electron donor and 8 μM cytochrome *c* as acceptor. The activity was fully inhibited by antimycin A at the concentration of 1 μg/ml.

Electrophoretic analysis of the polypeptide composition of native bc_1 complex and of its derivatives cross-linked with cytochrome c

Fig. 1 shows the subunit pattern of the purified bc_1 complex as obtained by scanning the unstained gel at 180 minus 300 nm directly after electrophoresis. The enzyme contained seven polypeptides with molecular weights as indicated in Table I. A very similar polypeptide distribution was obtained when the gel was stained with Coomassie blue and scanned at 570 minus 530 nm (not shown).

Scanning of the gel at 400 minus 430 nm (Fig. 1) showed that heme was

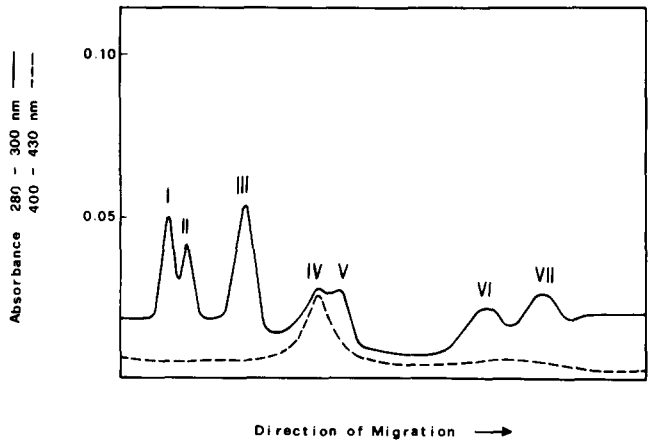

Fig. 1. Dodecyl sulfate polyacrylamide gel pattern of the isolated bc_1 complex as obtained by scanning at 280 minus 300 nm (solid line) and at 400 minus 430 nm (dashed line).

associated with only one band (band IV, molecular weight 30 000). This band can be attributed, therefore, to cytochrome c_1, since this is the only polypeptide in the enzyme containing covalently bound heme. Staining of the gels with tetramethylbenzidine gave the same result, further supporting the conclusion that band IV of the enzyme is cytochrome c_1. After the incubation and illumination of the bc_1 complex in presence of native cytochrome c, the enzyme underwent electrophoresis and the resulting gel was scanned at 280 minus 300 nm giving the pattern shown in Fig. 2a. No changes were observed in the high molecular weight region in comparison with the pattern presented in Fig. 1. A new polypeptide, having an apparent molecular weight of approx. 12 000 was also detected from its absorbance at 400 minus 430 nm and furthermore was stained by tetramethylbenzidine (not shown). This band, which was clearly cytochrome c, remained associated with the bc_1 complex or, alternatively, with lipids [20] present in the enzyme preparation and was not removed by passing down the Amberlite column.

When the bc_1 complex was incubated and illuminated with NAP-22-cytochrome c present (Fig. 2b) the same result was obtained as with native cytochrome c. No new band was formed, nor were other significant alterations of

TABLE I

MOLECULAR WEIGHTS OF bc_1 SUBUNITS AS ESTIMATED FROM POLYACRYLAMIDE DODECYLSULFATE GEL ELECTROPHORESIS

Polypeptide	Molecular weight ($\times 10^{-3}$)
I	53
II	50
III	38
IV cytochrome c_1	30
V	26
VI	13
VII	12

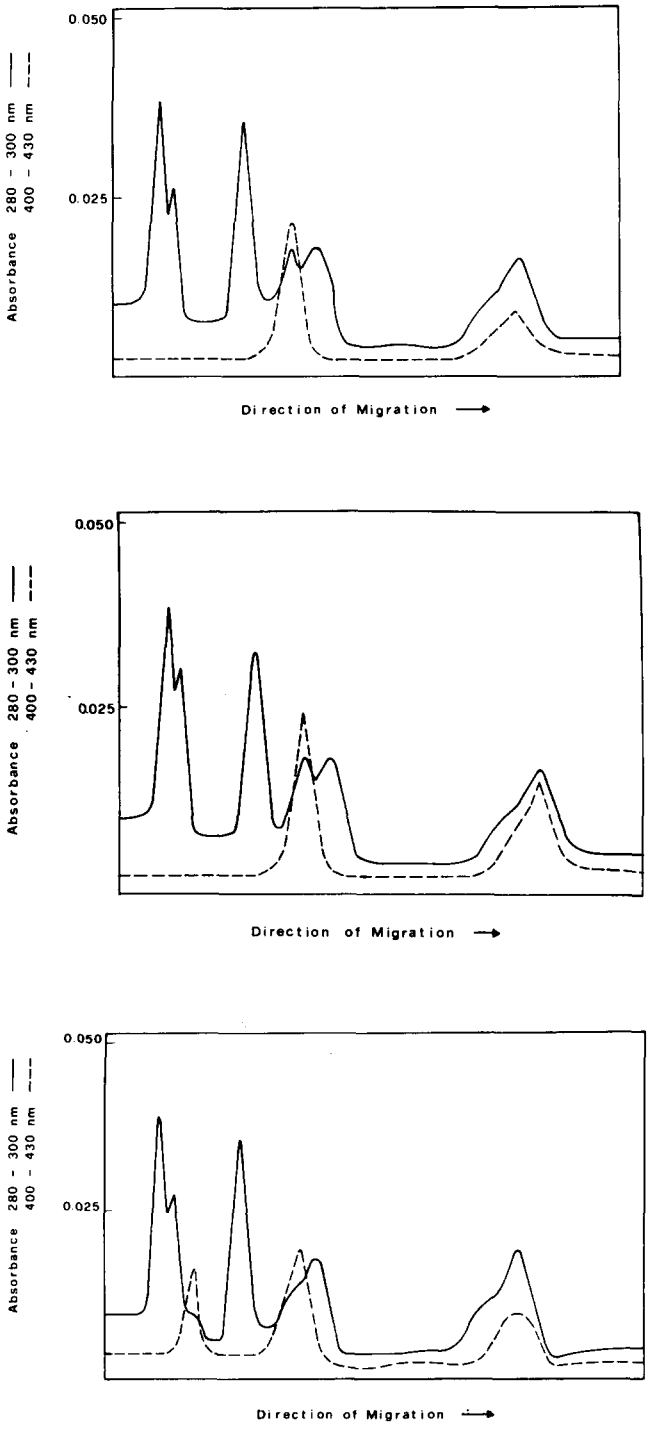

Fig. 2. Electrophoretic analysis and polypeptide composition of the bc_1 complex after illumination in the presence of: (a) native cytochrome *c*; (b) NAP-22-cytochrome *c*; (c) NAP-13-cytochrome *c*. Solid lines, scanning at 280 minus 300 nm. Dashed lines, scanning at 400 minus 430 nm. Experimental details are described in Materials and Methods.

the polypeptide pattern noticeable, suggesting that cytochrome *c* labeled at lysine 22 did not bind covalently to any subunit of the bc_1 complex.

Fig. 2c shows the subunit pattern of the bc_1 complex after incubation and illumination with NAP-13-cytochrome *c*. In the pattern obtained by scanning the gel at 280 minus 300 nm the band attributed to cytochrome *c* (as in Fig. 2a and 2b) is visible, but in addition a new band of apparent molecular weight of 45 000 appeared, while band IV (cytochrome c_1) was diminished. The new band absorbed also at 400 minus 430 nm, suggesting that it contained heme. It is important to note that the molecular weight of this new band is approximately equal to the sum of the molecular weight of band IV plus that of cytochrome *c*.

Discussion

Studies of the effects of lysine modifications on the interactions of cytochrome *c* with the bc_1 complex have provided a 'map' of the region of the cytochrome *c* surface involved in binding to the reductase. Ahmed et al. [8] found that, at high ionic strength, cytochrome *c* modified at lysine 22 has the same ability to accept electrons as the native cytochrome *c*, whereas modification of lysine 13 strongly inhibits electron transfer. It was concluded that lysine 13 is involved in the binding of cytochrome *c* to the bc_1 complex, whereas lysine 22 is not. A similar conclusion was reached by Speck et al. [9] and Rieder et al. [21]. From these studies it can also be concluded that the regions of cytochrome *c* involved in the binding to cytochrome *c* reductase or to cytochrome *c* oxidase overlap to a large extent. Experiments carried out to identify the polypeptide(s) of cytochrome *c* oxidase which interact(s) with cytochrome *c* have been undertaken with the use of photoaffinity labels covalently attached to the lysine-13 or lysine-22 residues of cytochrome *c*. In fact, these two residues have the peculiarity of reacting most readily with 4-fluoro-3-nitrophenylazide [11] and in addition, they are very useful in studying the interaction of cytochrome *c* with the oxidase or reductase since lysine 13 is in the center of the binding area whereas lysine 22 is outside it [9].

From the studies discussed above, it was expected that NAP-13-cytochrome *c* should cross-link with the bc_1 complex whereas NAP-22-cytochrome *c* should not.

Measurements of enzymatic activity of the bc_1 complex with cytochrome *c* derivatives as electron acceptors showed that there is no change in the functional properties of the derivatives. For all the types of cytochrome *c* used, the measured activities were identical. This is in agreement with Ahmed et al. [7] who found that a derivative at lysine 13 is as active as electron acceptor as native cytochrome *c*, if the enzymatic activity is measured at low ionic strength. In all cases the activity was inhibited completely by antimycin A. It can be inferred, thus, that the interactions of the bc_1 complex with the arylazido derivatives of cytochrome *c* are the same as those with native cytochrome *c*.

The formation of the bc_1-cytochrome *c* complex in the dark was studied using arylazido derivatives under a variety of experimental conditons, such as varying pH, ionic strength or concentration of cytochrome *c*. If the incubation

was performed at low ionic strength (5—10 mM Tris-HCl, pH 8.0) a large amount of cytochrome *c* was bound-non-specifically to the bc_1 complex. This resulted in the appeareance of a large band of molecular weight 12 000 following polyacrylamide gel electrophoresis of the complex. It is possible that a complex was formed between cytochrome *c* and phospholipids present in the bc_1 preparation. On the other hand, at higher ionic strength (around 25 mM Tris-HCl, pH 8.0) the preferential interaction of cytochrome *c* derivatives with the protein moiety of the bc_1 complex was obtained. This is in agreement with the observation of non-specific and specific binding of cytochrome *c* to the bc_1 complex at low and high ionic strength, respectively [6]. Since it was important to diminish the non-specific interaction of cytochrome *c* with the enzyme and its accompanying phospholipids and to reveal the specific interaction, higher ionic strength was used in further investigations.

Illumination of the dark complex between NAP-13-cytochrome *c* and the reductase resulted in the formation of a covalent product. This product could not be dissociated by high ionic strength or ion-exchange chromatography. The polypeptide profile of the complex obtained after polyacrylamide gel electrophoresis in the presence of sodium dodecyl sulfate, resulted in a specific diminution of band IV of the reductase (apparent molecular weight of about 30 000) and in the appearance of a new band (apparent molecular weight of about 45 000). This means that band IV (molecular weight 30 000) and cytochrome *c* (molecular weight 12 000) form a cross-linked product of molecular weight 45 000. This suggestion is supported by the evidence that the new band formed had the absorbance of a heme-containing polypeptide on scanning the gels at 400 minus 430 nm.

Since this is the only difference observed in the polypeptide profile of the bc_1 complex after cross-linking with NAP-13-cytochrome *c*, we conclude that band IV of the bc_1 complex is the binding site for cytochrome *c*. This band can be unequivocally identified as cytochrome c_1 from the fact it is the only one which absorbs at 400 minus 430 nm, and at 540 nm after staining with tetramethylbenzidine.

Illumination of bc_1 complex in presence of NAP-22-cytochrome *c* did not result in any change of the polypeptide pattern. This indicates that lysine 22 is not located in the region of binding of cytochrome *c* to the bc_1 complex. A similar result was found before, using the same photoaffinity label, for cytochrome *c* oxidase [11]. This is consistent with previous findings [9,21] that the sites of binding of cytochrome *c* with the oxidase and the reductase are very similar.

In conclusion, the results presented in this study indicate that cytochrome c_1 is the binding site of cytochrome *c* in the bc_1 complex.

Acknowledgements

This work was supported by the Swiss National Research Foundation (Grant No. 3.288.077), the Emil-Barrell-Stiftung and the Clark Joller Fund. C.B. is recipient of a fellowship from Schweizerische Gesellschaft für chemische Industrie and M.J.N. from UNESCO Contract SC/RP 551.861 and the Roche Foundation. We are grateful to Dr. R.P. Casey for helpful discussions.

References

1 Chance, B., DeVault, D., Legallais, V., Mela, L. and Yonetani, T. (1967) Nobel Symposium, Vol. 5 (Claesson, S., ed.), pp. 437—468, Interscience, New York
2 Dutton, P.L., Wilson, D.F. and Lee, C.P. (1970) Biochemistry 9, 5077—5082
3 Chance, B. (1957) Methods Enzymol. 4, 273—329
4 Rieske, J.S. (1976) Biochim. Biophys. Acta 456, 195—247
5 Bosshard, H.R., Zürrer, M., Schägger, H. and von Jagow, G. (1979) Biochem. Biophys. Res. Commun. 89, 250—258
6 Weiss, H. and Juchs, B. (1978) Eur. J. Biochem. 88, 17—28
7 Chiang, Y.L., Kaminsky, L.S. and King, T.E. (1976) J. Biol. Chem. 251, 29—36
8 Ahmed, A.J., Smith, H.T., Smith, M.B. and Millet, F.S. (1978) Biochemistry 17, 2479—2483
9 Speck, S.H., Ferguson-Miller, S., Osheroff, N. and Margoliash, E. (1979) Proc. Natl. Acad. Sci. U.S.A. 76, 155—159
10 Bayley, H. and Knowles, J.R. (1977) Methods Enzymol. 46, 69—114
11 Bisson, R., Azzi, A., Gutweniger, H., Colonna, R., Montecucco, C. and Zanotti, A. (1978) J. Biol. Chem. 253, 1874—1880
12 Blair, P.V. (1967) Methods Enzymol. 10, 78—81
13 Hatefi, Y. and Rieske, J.S. (1967) Methods Enzymol. 10, 225—231
14 Rieske, J.S. (1967) Methods Enzymol. 10, 239—245
15 Wan, Y.P. and Folkers, K. (1978) Methods Enzymol. 53, 591—599
16 Harbury, H.A. and Loach, P.A. (1960) J. Biol. Chem. 235, 364—3645
17 Weber, K. and Osborn, M. (1969) J. Biol. Chem. 244, 4406—4412
18 Broger, C., Allemann, P. and Azzi, A. (1980) J. Appl. Biochem, in the press
19 Brautigan, D.L., Ferguson-Miller, S. and Margoliash, E. (1978) J. Biol. Chem. 253, 130—139
20 Nicholls, P. (1974) Biochim. Biophys. Acta 346, 261—310
21 Rieder, R. and Bosshard, H.R. (1978) FEBS Lett. 92, 223—226