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The ADP/ATP carrier from yeast (AAC-2) is uniquely suited for the assignment of the binding center by photoaffinity labeling

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The ADP/ATP carrier from yeast was photoaffinity-labeled in mitochondria with 2-azido-[α-^{32}P]ATP in a binding-center-specific, i.e. carboxyatractylate-sensitive, manner. After isolation, fragmentation possibilities unique for the yeast AAC-2 could be exploited to assign the insertion to a narrow range of the sequence. The CNBr fragment 115–210 contained all the incorporated label which corresponds to the second domain within the triple-domain primary structure of the AAC. With hydroxylamine cleavage directed to the Asn 171-Gly 172 site, all the label was found in the C-terminal 16 kDa fragment. Thus the 2-azido-ATP incorporation is clearly delimited to the 172–210 segment. 8-Azido-[α-^{32}P]ATP could be site-specifically incorporated only in isolated AAC since it has a much lower affinity for AAC than 2-azido-ATP. The label was also exclusively found in the 172–210 region. With both forms no incorporation into the C-terminal region was found, as claimed for bovine AAC. The labeled segment contains Lys 179 and 182 which are homologous to bovine Lys 162 and 165 and which have been proposed to be in the translocation path.

ADP/ATP carrier; Photoaffinity labeling; Azido-ATP, 2-; Azido-ATP, 8-; (*Saccharomyces cerevisiae*)

1. INTRODUCTION

Efforts to characterize the binding center of the ADP/ATP carrier (AAC) by covalent labeling have been continued for considerable time. 3′-Arylazido-ATR was the first photoaffinty label of which an insertion was localized in a region between residues 154 and 200 in the bovine heart AAC [1]. With the advent of 2-azido-ADP, the binding was found in the same center region of bovine AAC, in addition to some incorporation at the C-terminal region [2]. In a different approach, using pyridoxal phosphate as a covalent probe for lysine groups, Lys 22 and, as part of the translocation path, Lys 162 and 165 in bovine AAC were implied to be the residues linked to the binding center [3]. The same residues were assigned by marginal evidence to 2-azido-ADP binding.

Recently [4], we purified AAC from yeast and characterized its CNBr segmentation pattern in accordance with the primary structure of the second yeast AAC (AAC-2) obtained by cDNA sequencing [5]. A great advantage of the yeast AAC-2 is the possibility to obtain a CNBr fragment containing only the central domain within the tripartite sequence of AAC, whereas with mammalian AAC and also with AAC-1 from yeast this is not possible. Therefore, using CNBr as the cleanest cleavage procedure, a much better definition of incorporation can be achieved than before. For this reason, we investigated the incorporation of 2-azido-ATP searching for a more unique assignment of its localization than so far attained. Moreover, we succeeded in localizing the specific incorporation of 8-azido-ATP in AAC, which has not yet been reported.

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Abbreviations: $C_{10}E_5$, *n*-decylpentaoxyethylene; $C_{12}E_8$, *n*-dodecyloctaoxyethylene; octyl-POE, *n*-octylpolyoxyethylene; PMSF, phenylmethylsulfonyl fluoride; SDS-PAGE, SDS-polyacrylamide gel electrophoresis; CAT, carboxyatractyloside; YWM, yeast wildtype mitochondria

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2. MATERIALS AND METHODS

$H_3{}^{32}PO_4$ was purchased from New England Nuclear. Triton X-100 and 8-azido-ATP were from Sigma, $C_{10}E_5$ from Bachem and $C_{12}E_8$ from Fluka. Octyl-POE was a gift from Professor G. Rosenbusch. Zymolyase was purchased from Miles Scientific. All other reagents used were purchased as the purest grade available. The yeast strain used in this study was *S. cerevisiae* D 273 10 B α. Conditions for cell growth and isolation of mitochondria are outlined in [6,7].

2.1. *Synthesis of 2-azido-[α-^{32}P]ATP and 8-azido-[γ-^{32}P]ATP*

2-Azido-adenosine was synthesized as in [8], 2-azido-[α-^{32}P]AMP was synthesized as described in [9], except for the fact that the purification was replaced by chromatography on DEAE-cellulose with a linear gradient of triethylammonium-HCO_3 (0.1–0.5 M). Phosphorylation to 2-azido-[α-^{32}P]ATP was carried out as in [10]. The purified product usually had a specific activity of 5000 dpm/pmol and was diluted with nonlabeled 2-azido-ATP prior to the incubation with mitochondria.

8-Azido-[γ-^{32}P]ATP was synthesized by enzymatic exchange with $H_3{}^{32}PO_4$. The incubation conditions used are outlined in [11], with the modifications, that the concentration of 8-azido-ATP was lowered to 1.3 mM and 13 mCi $H_3{}^{32}PO_4$/ml was used. The reaction was stopped by heating to 78°C for 5 min. The yield was usually about 80% with respect to incorporated radioactivity. The product was used without further purification.

2.2. *Photoaffinity labeling of YWM with 2-azido-[α-^{32}P]ATP*

30 min prior to irradiation with UV-light the yeast mitochondria (5 mg protein) were incubated with 50 μM 2-azido-[α-^{32}P]ATP (spec. act. 2500 dpm/pmol) in 400 μl medium containing 600 mM mannitol and 20 mM Tris-HCl, pH 7.4. For the control the mitochondria were preincubated with 30 μM CAT.

The probes were irradiated with a 90 W Hg lamp. To protect AAC against photooxidation and UV damage, the probes were flushed with N_2 and a chloroform filter (1 cm) was installed. After 10 min irradiation at 4°C the sample was centrifuged. The pellet was washed three times by resuspension in the incubation medium supplemented with 2 nmol CAT/mg mitochondrial protein to separate noncovalently bound label and to bring the nonlabeled AAC into the more stable CAT-bound form.

2.3. *Purification of the radiolabeled AAC*

For purification of AAC the pellet was dissolved in a medium with octyl-POE, detergent/protein ratio = 2, 20 mM Mops, 0.5 mM PMSF, 0.5 mM EDTA, pH 7.4. After centrifugation, the pellet was resuspended in a medium containing either $C_{10}E_5$ or Triton X-100 at a detergent/protein ratio of 2, 500 mM ammonium acetate and 0.5 mM PMSF, pH 7.4. The extract was thoroughly mixed with prewashed hydroxyapatite (250 mg/mg protein) for 5 min [12]. After centrifugation the supernatant was precipitated with trichloroacetic acid/acetone yielding essentially pure AAC.

2.4. *Photoaffinity labeling of the solubilized AAC with 8-azido-[γ-^{32}P]ATP*

For photoaffinity labeling of the solubilized purified AAC with 8-azido-[γ-^{32}P]ATP, AAC was first purified from unlabeled mitochondria as described above except that $C_{12}E_8$ instead of $C_{10}E_5$ was used. The isolated protein was incubated with 8-azido-[γ-^{32}P]ATP (6.5 mol label/mol protein and a spec. act. of 5000 dpm/pmol) in the isolation buffer, supplemented with 0.5 mM DTE, for 10 min. Irradiation with UV-light and precipitation of the labeled protein was carried out as above.

2.5. *Chemical cleavages*

Cleavage at the methionyl residues was performed in 80% formic acid overnight with CNBr using 500-fold molar excess over the methionines present [13]. The reaction was stopped by dilution with water (1:10) and the sample was freeze-dried. Cleavage at the Asn-Gly bond by hydroxylamine was carried out as described in [14], except that the protein was dissolved in 1% SDS instead of in guanidinium-HCl and a pH of 10 was used.

Labeled mitochondria were separated by polyacrylamide gel electrophoresis using 12.5% polyacrylamide [15]. For separation of peptides obtained from chemical cleavages, the gel system developed by Schägger and Von Jagow [16] was used. For autoradiography, the gels were exposed to a Fuji RX-film at −80°C overnight.

3. RESULTS

The incorporation of 2-azido-[α-^{32}P]ATP was first applied to AAC still localized in yeast mitochondria. The specificity of the incorporation was controlled by also using mitochondria in which AAC has been liganded with the tightly binding inhibitor CAT. The photoaffinity labeled mitochondria were extracted with SDS and subjected to polyacrylamide gel electrophoresis. As shown in fig.1, the protein bands of mitochondria are juxtaposed to the autoradiography of the same gel. The results show that by far the main band labeled by 2-azido-[α-^{32}P]ATP is localized at 31 kDa in the same region as the yeast AAC. The specificity of labeling to AAC and also to the binding center within AAC is clearly demonstrated by the virtually complete inhibition of 2-azido-ATP incorporation into CAT-loaded mitochondria (fig.1) since it is held that ATP and CAT bind to a common site [17]. Thus these incubation and photoaffinity labeling conditions are suitable for the specific labeling of the AAC-binding center.

For the purification of radiolabeled AAC it was necessary to develop a rapid and simple isolation procedure, since AAC not protected by CAT is easily degraded by proteases in nonionic detergents. Therefore the previously published method [18] for extracting the ADP/ATP carrier

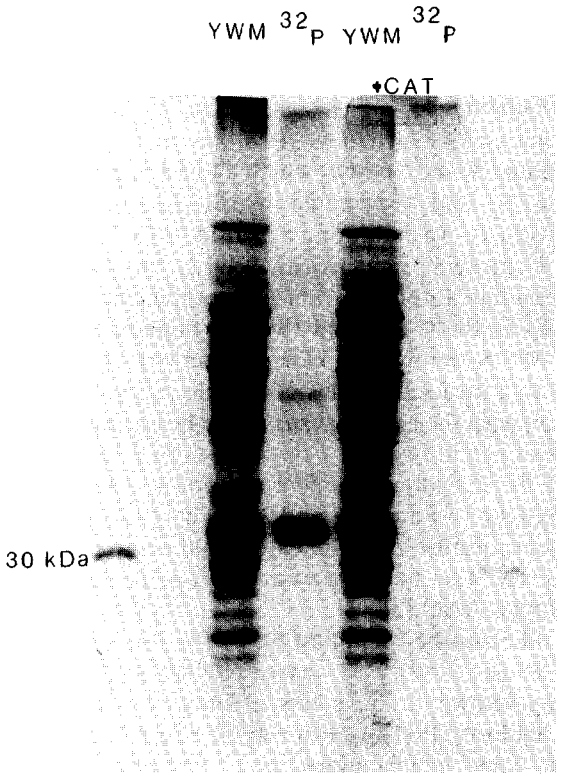

Fig.1. SDS-PAGE separation of yeast wildtype mitochondria (YWM) labeled with 2-azido-[α-^{32}P]ATP and the corresponding autoradiography pattern. Radiolabeled mitochondria were extracted with 3% SDS and applied to SDS-PAGE using 12.5% polyacrylamide. For the control, mitochondria preincubated with CAT were treated equivalently and also applied to SDS-PAGE. As molecular mass standard AAC from beef heart mitochondria (30 kDa) was used.

had to be modified. As already recently mentioned, the major problem in purifying the ADP/ATP carrier from yeast is the contamination by a major proportion of porin with a molecular mass of 29 kDa after passing through hydroxyapatite [4]. For this reason, the mitochondria were first preextracted since, based on previous experience with beefheart mitochondria, octyl-POE at low salt concentration removes most of the porin together with the majority of other mitochondrial proteins, while the major part of AAC remained in the sediment after ultracentrifugation. The best results were obtained with an octyl-POE/mitochondrial protein ratio = 2 in a low salt containing buffer. This pellet was then resuspended in a high salt medium containing either Triton X-100 or $C_{10}E_5$, together with 0.5 M ammonium acetate. For a more rapid purification instead of using a hydroxyapatite column, the extract was thoroughly mixed with the pretreated hydroxyapatite paste according to the 'batch procedure' [12]. After centrifugation, the supernatant contained essentially pure AAC as indicated by polyacrylamide gel electrophoresis of the trichloroacetic acid/acetone precipitate (fig.2).

For localizing the insertion of 2-azido-ATP into the primary structure, purified AAC was subjected to two different cleavage procedures. First, the CNBr cleavage was applied which produces fragments unique for AAC from yeast, i.e. a fragment of about 10 kDa which largely corresponds to the central domain [4]. It was a gratifying finding, as shown in the autoradiogram of the CNBr fragment in fig.2, that nearly all of the radioactivity was concentrated in this particular 10 kDa fragment. This result assigns the incorporation of the radiolabeled 2-azido-ATP exclusively to the position between 115 and 210.

In order to further delimit the position of the radiolabel, hydroxylamine cleavage [14] was ap-

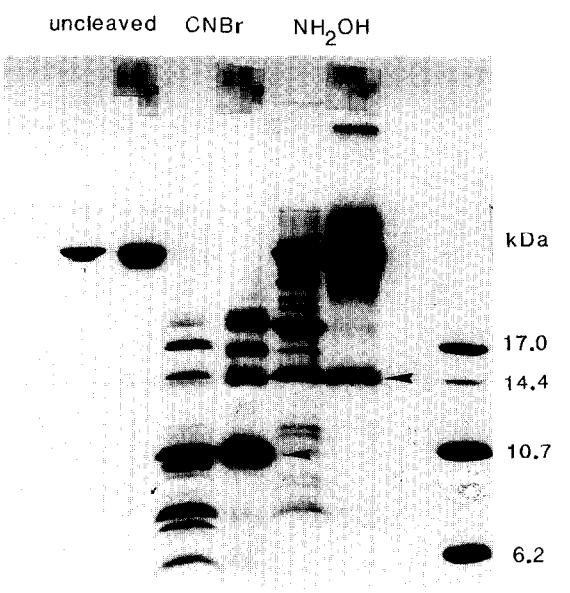

Fig.2. SDS-PAGE of isolated AAC labeled with 2-azido-[α-^{32}P]ATP and separation of the fragments obtained by cleavage with CNBr at Met and with NH_2OH between Asn-Gly, respectively, and the corresponding autoradiography. The gel system described in [16] was used. Molecular mass standards are CNBr fragments of horse heart myoglobin.

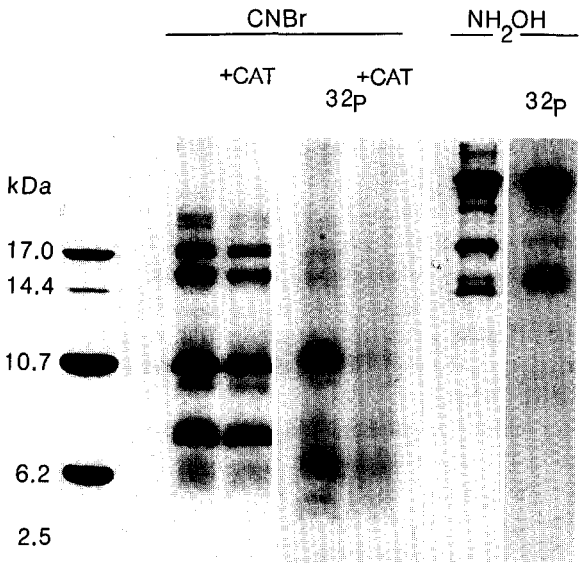

Fig.3. SDS-PAGE of the cleavage products of isolated AAC photolabeled with 8-azido-[γ-^{32}P]ATP obtained by CNBr and NH_2OH treatment and the corresponding autoradiography. To prove the specificity of the incorporation of 8-azido-ATP into purified AAC, the CAT-AAC complex was also fragmented with CNBr after treatment with 8-azido-[γ-^{32}P]ATP. Molecular mass standards are CNBr fragments of horse heart myoglobin.

plied to the whole protein since in AAC-2 a unique hydroxylamine cleavage site is localized at Asn-171/Gly-172 within the central 10 kDa CNBr fragment. This cleavage produces, apart from some minor products, two major fragments of about 19 and 16 kDa which should correspond to the N- and C-terminal hydroxylamine cleavage products. In addition, a considerable portion of the protein remains uncleaved. In the corresponding autoradiogram the isotope label is found only in the C-terminal 16 kDa fragment and none in the N-terminal 19 kDa product (fig.2). By combining the localizations of 2-azido-ATP incorporation with the two cleavage procedures, one arrives, as shown in fig.4, at a clearly delimited assignment of the 2-azido-ATP incorporation to the segment between residues 172 and 210. There is no trace of incorporation near the C-terminal, as was described for the incorporation of 2-azido-ATP into bovine AAC [2].

For another delimitation of the binding center, 8-azido-[γ-^{32}P]ATP was employed. 8-Azido-ATP was known to have less affinity for AAC than 2-azido-ATP [19]. However, it has the advantage of being much more easily brought into the

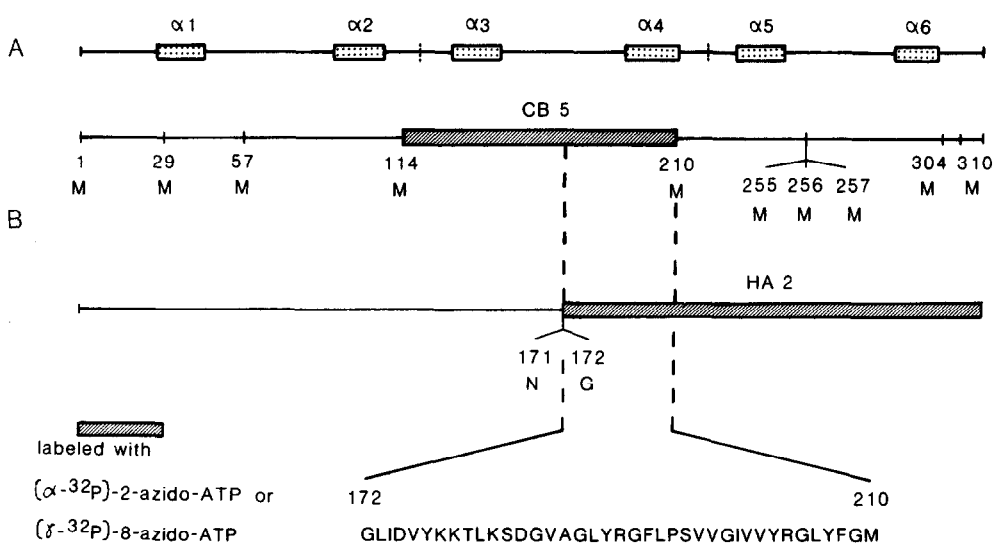

Fig.4. (A) Scheme illustrating the tripartite structure of AAC. The marked areas correspond to the putative hydrophobic transmembrane-spanning helices. (B) Strategic amino acid position for CNBr and NH_2OH cleavage and localization of incorporated 2-azido-[α-^{32}P]ATP and 8-azido-[γ-^{32}P]ATP corresponding to the AAC-2 sequence [5] in a region between Gly 172 and Met 210. The labeled range overlaps with parts of the hydrophilic center region and with the helix α4.

radioactive form by enzymatic exchange of $^{32}P_i$ with the commercially available 8-azido-ATP. Attempts to label AAC in yeast mitochondria with 8-azido-ATP, however, failed. No incorporation was observed either to AAC or to other ATP-binding proteins such as the ATP-synthase. We therefore applied photoaffinity labeling with 8-azido-[γ-^{32}P]ATP to a suitably isolated AAC. For a better stabilization of the unliganded AAC, after preextraction of porin with octyl-POE, AAC was solubilized with $C_{12}E_8$ in the presence of ammonium acetate (Krämer, R., personal communication). As shown in fig.3, a significant incorporation into solubilized AAC was indeed found. Importantly, this incorporation was nearly fully suppressed when the CAT-AAC was exposed to 8-azido-[γ-^{32}P]ATP.

The CNBr cleavage of the 8-azido-ATP labeled protein shows incorporation primarily into the 10 kDa fragment, similar to the observation with 2-azido-ATP (fig.2). The low-level labeling of smaller fragments seems to be unspecific since it is not suppressed by CAT (fig.3). The hydroxylamine cleavage was also applied to the 8-azido-ATP-labeled AAC. It clearly demonstrates that 8-azido-ATP too is primarily incorporated into the C-terminal 16 kDa cleavage product (fig.3) with only a minor incorporation into the N-terminal 19 kDa fragment. Again a considerable portion of AAC remains uncleaved. We can conclude that 8-azido-ATP is incorporated into AAC in a confined region between residues 172 and 210, just like 2-azido-ATP (fig.4).

4. DISCUSSION

The affinity labeling of the ADP/ATP carrier from yeast takes advantage of particular cleavage opportunities which are provided in the primary structure of AAC-2. The unique delimitation of the affinity binding to a region between residues 172 and 210 is facilitated by the occurrence of Met-114 for CNBr cleavage and of Asn-171/Gly-172 for hydroxylamine cleavage. According to the tripartite model of AAC in which the primary structure can be divided into three, about equally long, somewhat homologous domains [20–22], each domain contains a central, more highly conserved and more hydrophilic region and two less conserved hydrophobic putative transmembrane-spanning α-helices (fig.5) [23]. According to the folding model of AAC, as derived in part from pyridoxal labeling of lysine groups [3], these hydrophilic center regions are localized more in the m-region and partially reach through the membrane along the hydrophilic translocation path. The 2- and 8-azido-ATP-labeled region between 172 and 210 overlaps with parts of the hydrophilic center region and with the second α-helix. Previously, for bovine heart AAC, Lys 162 and 165 have been assigned to the translocation path because they are accessible also from the c-side to pyridoxal phosphate [3]. Both these residues are conserved as Lys 179 and 182 in yeast AAC-2.

In bovine heart mitochondria the incorporation of 2-azido-ADP was found to be localized in the homologous region between residues 153 to 200 [2]. Within these peptides a diffuse incorporation into the various residues was observed with minor excesses at Lys 162, Lys 165 and Ile 183. Dalbon et al. [2] conclude that part of this region including Lys 162 and Lys 165 are localized at the c-surface and therefore the binding center is located more towards the c-side. This is in line with the model considered by the these authors that there may be two binding sites along the translocation path. In this context our localization data for Lys 162, Lys 165 are misinterpreted since they postulate an access from the c-side through the translocation path.

We maintain that any membrane folding model for the assignment of the photoaffinity labeling should take into account that there is a single-binding site in the AAC molecule and therefore this binding site should be localized more in the central part of the protein within the membrane-spanning sections. The observed labeling of the central sections would concur with this model by assuming that part of these sections are folded through the membrane.

In contrast to the findings with yeast AAC, in bovine AAC a major portion of 2-azido-ADP is reported to be incorporated into a second region near the C-terminal between residues 250 and 281. This has been interpreted as due to significant mobility of the adenine moiety or, which seems more probable, as a contact region to the neighbouring subunit. In view of the fact that the incorporation into yeast AAC is unique to only

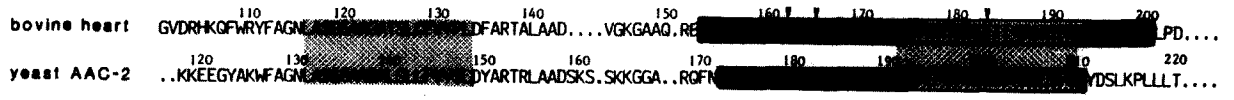

Fig.5. Comparison of the second domain within the primary structure of bovine heart AAC [24] and yeast AAC-2 [5]. Putative helical areas are accentuated and the marked regions of the sequences correspond to the photoaffinity binding regions within the second domain of bovine heart AAC [2] and yeast AAC-2. Lys 162, Lys 165 and Ile 183 reported to be predominantly labeled by 2-azido-ADP in bovine heart AAC [2], are marked with arrowheads.

one region, we may also suspect that the incorporation into the C-terminal region in bovine AAC is unspecific.

The uniqueness of the central section for binding 2-azido-ATP is emphasized by the incorporation also of 8-azido-ATP exclusively to the same region. This finding is also significant in view of the *anti*- and *syn*-conformations stabilized either in the 2- or 8-azido-ATP. The preferred *anti*-conformer 2-azido-ATP has a higher affinity for AAC than ADP or ATP and obviously better fits the binding center. Probably for this reason the preferred *syn*-conformer 8-azido-ATP has a much lower affinity. In their opposite conformations both 2- and 8-azido-ATP expose the azido group to the same side with respect to the ribose moiety and this may rationalize the labeling by both derivatives of the same region.

A more precise localization of the azido-ATP incorporation is desirable. However, it should be stated that in our experience sequencing of azido-ATP-labeled peptides is extremely difficult. It is virtually impossible to handle in a gas-phase sequencer because the ADP,ATP-substituted amino acids are not extractable during the Edman degradation. In solid-phase sequencing artefacts are seen by the enormous 'bleeding' of the radioactive label (unpublished results and see fig.7 in [2]).

Since the genetic manipulation of AAC in yeast is fairly straightforward, yeast offers a favourable platform for research into the molecular biology of AAC. This work on the approximate assignment of the binding center within the primary structure also provides a basis for future work by other approaches, such as site-directed mutagenesis for the elucidation of the carrier mechanism.

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