DIFFERENCES IN α-AMINO ACETYLATION OF ISOZYMES OF YEAST ALCOHOL DEHYDROGENASE

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1. Introduction

Native proteins and peptides often have acetyl-blocked α-amino groups. This N-terminal modification was first discovered in a viral coat protein [1] and a hormonal peptide [2] but is now known to be very common, affecting many classes of naturally occurring polypeptide chains [3]. The proteins are usually recovered in completely blocked form [3], and the modification is enzymatically performed on the nascent polypeptide chain with acetyl-CoA [4]. The blockage can be prevented during translation in cell-free systems by artificial removal of acetyl-CoA [5]. Once attached, however, the N-α-acetyl group is comparatively inert [6]. The functional significance of acetylation is unknown, but it has been suggested that it may protect against premature protein catabolism [3].

Yeast alcohol dehydrogenase normally has an acyl-blocked N-terminus [7]. Acetylation of this protein was therefore likely but not directly shown until the present report. During studies of the two major isozymes, it was discovered that the N-terminus of the protein subunits may also be recovered in unblocked form under certain physiological conditions [8]. Although isozyme separations were incomplete, the presence of unblocked molecules appeared to be associated with growth conditions and isozyme patterns. Since both blocked and unblocked (acetylated and unacetylated) molecules have activity, this finding provided an opportunity to investigate the possible roles of acetylation in protein metabolism and enzyme function.

We show here first that both isozyme I and II of yeast alcohol dehydrogenase are indeed normally acetylated. The two isozymes are found to be blocked to different degrees in the same cell. The amount of blockage is a function of the growth conditions but does not affect enzyme activity. Most but not all of the unblocked enzyme is newly synthesized. Consequently, unblocked enzyme seems more to be due to absence of acetylation than to deacetylation under these physiological conditions. Finally, two new amino acid differences between isozymes I and II have been found.

2. Materials and methods

The relative amounts of acetylation were investigated in isozymes from the same preparation. Strain X2180-1A, mating type *a*, of *Saccharomyces cerevisiae* was grown to plateau in a New Brunswick Microferm fermentor with vigorous aeration (4 scf/h). The medium used was 10 l 1% yeast extract, 2% Bacto-Peptone, 2% dextrose, all from Difco. The cells were collected by centrifugation, washed with potassium phosphate buffer (0.05 M, pH 7.0), ground with an equal weight of alumina, and extracted in 0.1 M potassium phosphate buffer (pH 7.0). A protamine sulfate precipitation [8] of nucleic acids and a fractionated acetone precipitation at −2°C [9] were then performed (the latter between 40–110% acetone in relation to the original supernatant volume).

The final acetone precipitate was resuspended in 10 mM potassium phosphate (pH 7.0), 10 mM mercaptoethanol, and applied to a column of 100 ml Cibachrom blue F3GA/dextran (Affi-Gel blue from BioRad). The gel had been washed with 6 M urea, 2 M NaCl and 10 mM EDTA before equilibration with the starting buffer. After sample application

and washing with the starting buffer, the alcohol dehydrogenase was eluted with a NADH (0–80 μM) 500 ml gradient. NADH was freshly purified [10,11] and had an A_{260}/A_{340} of <2.3. Isozyme II, which has a greater affinity for NADH [11], was eluted first, as was determined by gel electrophoresis and activity staining. Three fractions were obtained: pure isozyme II; a mixture of II and I; and pure isozyme I. Since the cells had been grown aerobically, most of the enzyme in the preparation was isozyme II. The pure fractions had spec. act. 292 and 299 U/mg at pH 8.8, 23°C, where 1 U is the reduction of 1 μM NAD/min. SDS–polyacrylamide slab gel electrophoresis revealed no detectable impurities in any of the 3 fractions. Total yield was 60%.

The salt-free and freeze-dried proteins were directly applied to a liquid-phase sequencer (Beckman 890C) to estimate the extent of free N-terminal residues. Degradations with the 1 M quadrol protein program were performed in the presence of polybrene, pretreated for 2 cycles, and the PTH-amino acids were identified by high performance liquid chromatography (Hewlett-Packard 1084B) plus thin-layer chromatography [12]. In the isozyme mixture, the two isozymes could be distinguished because of differences in positions 8 and 9.

For identification of the blocking acyl groups isozymes from different preparations were used, pure isozyme I from respiration-deficient cells and 85% pure isozyme II from respiration-competent cells (detailed in [8]). The N-terminal tryptic peptides (called T1 in [7]) were prepared on paper, using high-voltage electrophoresis and chromatography as in [8]. The freeze-dried heptapeptides from both preparations (300 nmol) were cleaved with staphylococcal protease I (50 μg in 500 μl 0.1 M ammonium bicarbonate, 4 h, 37°C), which in both cases liberated the blocked tetrapeptide (acyl–Ser–Ile–Pro–Glu) from the original tryptic peptide [7]. This fragment was purified by Dowex 50W-X2 chromatography [13]. It was found to have the expected total composition, and revealed no detectable N-terminus with the dansyl method.

The freeze-dried blocked tetrapeptides were esterified using diazomethane, and the methyl esters were analyzed on a Finnigan 1015 mass spectrometer equipped with a PDP 8/e computer, a system 150 interface and a Houston plotter. The samples were introduced into the source through a solid inlet probe, which was heated to 250°C within 3–4 min. The source was maintained at 225°C and the ionizing voltage was 70 eV.

3. Results

3.1. *Mass-spectrometric analysis of the N-terminal regions of isozymes I and II*

The blocked N-terminal tetrapeptide from isozyme I and the same peptide from the isozyme II preparation were both methylated and analyzed in the mass spectrometer after introduction (15–25 μg) through the solid inlet probe [14,15]. Resulting peaks and their identifications are shown in table 1. Both sam-

Table 1
Major sequence determining ions from mass-spectrometric analysis of the tetrapeptides acyl-Ser–Ile–Pro–Glu(OMe)$_2$ derived from both isozymes I and II

Peak	Identification	Peak	Identification
514	acetyl-Ser–Ile–Pro–Glu(OMe)$_2$ (M^+)	130	acetyl-Ser
483	M–(OCH_3)	113	HN–CH–CO C_4H_9
471	M–($COCH_3$)	112	CH_3CO–NH–C–CO CH_2
455	M–($COOCH_3$)	102	CH_3–CO–NH–C CH_2OH
384	M–acetyl-Ser	97	N———CO (from Pro)
355	acetyl-Ser–Ile–Pro–NH	71	CH_3–CO–NH–CH
340	acetyl-Ser–Ile–Pro	58	CH_3–CO–NH
312	acetyl-Ser–Ile–Pro–(CO)	43	CH_3CO
243	acetyl-Ser–Ile–		
215	acetyl-Ser–Ile–(CO)		
174	HN–CH–$COOCH_3$ CH_2–CH_2–$COOCH_3$ (from Glu)		
145	acetyl-Ser–NH		

ples yielded identical peaks, except for minor deviations in relative intensities, due to slight differences in the magnitudes of fragmentation.

Peaks at *m/z* 43 (CH_3CO), 58 (CH_3CONH), 71 ($CH_3CONHCH$), 130 ($CH_3CONHCH$-(CH_2OH)CO) and 471 (molecular ion, CH_3CO), as well as the peaks corresponding to the acetylated fragments in table 1, identify the peptides as acetylated. *m/z* 71 is the N-terminal substitutent-determining ion, X-NHCH [16], of the peptide derivative. No further, unexplained low-molecular weight peaks compatible with other acyl groups were identified from either peptide. Consequently, the blocking group is concluded to be acetyl in both isozymes. In addition, this analysis confirmed the known amino acid sequence (Ser–Ile–Pro–Glu [7]) of both peptides as shown in table 1.

3.2. *Analysis of free N-terminal regions of isozymes I, II and the mixture I/II*

The 3 preparations were analyzed in a Beckman 890C sequencer in the presence of polybrene, as given in section 2. The results are shown in table 2. It was found that preparations are clean and that none was completely without a free α-amino group. Background build-up of PTH-derivatives took place gradually (starting to interfere after about cycle 10). This may be related to the unfavourable stoichiometry of PTH-derivative towards protein chain due to the N-terminal blocking group.

The positions analyzed in isozyme I were reported in [7,8]. Isozyme II, however, was found to differ at two positions in the N-terminal region: alanine in position 8 (instead of glycine in isozyme I), and isoleucine in position 9 (instead of valine in isozyme I).

Table 2
Results of sequence degradations of 3 different isozyme preparations of yeast alcohol dehydrogenase

Cycle	Mixture I + II		Isozyme II			Isozyme I		
	Residue	Recovery (nmol)	Residue	Recovery		Residue	Recovery	
				(nmol)	(%)		(nmol)	(%)
1	Ser	5	–			–		
2	Ile	20	Ile	7	130	Ile	2	40
3	Pro	15	Pro	6	140	Pro	2	50
4	Glu	15	Glu	6	120	Glu	2	50
5	Thr	10	–			–		
6	Gln	15	Gln	5	110	Gln	2	40
7	Lys	20	Lys	6	100	Lys	1	20
8	Ala (Gly trace)	20	Ala	6	120	(Gly)	1	20
9	Ile (Val trace)	15	Ile	7	140	(Val)	3	60
10	Ile	20	Ile	6	110	Ile	3	50
11	Phe	20	Phe	6	100	Phe	1	20
12	Tyr	15	Tyr	5	110	Tyr	2	30
Average PTH-recovery[a] as % of the amount of protein		15		20			5	

[a] Excluding the hydroxy acid derivatives in cycles 1 and 5

The isozyme mixture analyzed totalled 130 nmol and contained ~50% isozyme I and 50% isozyme II as judged by gel electrophoresis. The two separate isozymes analyzed were 90–100% pure and consisted of ~35 nmol each. Recoveries of >10 nmol are given to the nearest integer of 5. Recoveries in % for the separate isozymes indicate yields in relation to an identical amount of the isozyme mixture

Because of these sequence differences, the isozyme which was unblocked in each sample could be determined, along with the extent to which it was unblocked. Although initial yields of coupling in sequencer analyses depend on many conditions, recoveries are usually ≥50–75%, even on samples more harshly pretreated than the present proteins [12,17]. Direct comparisons of the amounts of PTH-derivatives in identically-pretreated samples, as here, are valid, and the results obtained from the individual isozymes agree with those from the mixture.

Isozyme II residues were obtained in yields (table 2, bottom of middle column) that suggest the initial degree of blocking to be maximally 80%, and probably about 60% (considering the recovery in initial coupling). Isozyme I, however, is blocked to 90–95% (table 2, last column), and probably even more, since the small yields for the residues from this isozyme tend to be overestimated because of background interference. As expected, the mixture (table 2, first column) gave an intermediate yield, and had the unblocked chains mainly of the isozyme II type as revealed by the deviating residues in positions 8 and 9.

4. Discussion

4.1. *N-terminal blocking group in yeast alcohol dehydrogenase*

The mass spectrometric data establish that the α-amino acyl group detected in alcohol dehydrogenase [7] is indeed an acetyl group and that this is present both in isozyme I and in isozyme II (table 1). Yeast alcohol dehydrogenase is therefore a truly acetylated protein. The sequence analysis, however, also confirms indications that the acetyl blocking is not stoichiometric [8], and that it may be variable.

4.2. *Type of isozyme acetylated and extent of modification*

Analysis of the pure isozymes establish that it is isozyme II that attributes most of the free α-amino group. This isozyme is estimated to have 20–40% unblocked protein chains. These values are somewhat larger than the calculation of ~15% unblocked chains in another isozyme mixture [8]. However, the possibility is not excluded by that study that the unblocked form might have been isozyme I can now be disregarded. The main unblocked form is isozyme II, both in the pure isozymes and in the mixture (table 2). Affinity chromatography did not separate the blocked and unblocked forms of either isozyme.

Isozyme I is ≥4-fold more extensively blocked in the pure preparation as well as in the mixture. This means acetylation to ≥90%, close to complete α-amino substitution. Significantly, however, non-acetylated chains are still detectable even in this isozyme. These free chains are not contaminants of isozyme II since the residues in positions 8, 9 show that the alternatives are those expected for isozyme I.

4.3. *Functional significance of N-terminal acetylation*

No coupling is apparent between alcohol dehydrogenase activity and acetylation. The pure isozyme II preparation, which may be non-acetylated up to 40%, is just as active as the almost completely acetylated isozyme I preparation. The fact that the blocking has no effect on affinity-column binding also suggests that the active sites are unimpaired. Acetylation can therefore not be a requirement for the enzyme activity and probably has no significance for the structure–function relationship of the protein. This conclusion is in full agreement with the generally superficial positions of acetylated N-terminal residues, as noted [3].

It is now established that it is the newly synthesized protein (isozyme II, induced by aerobic conditions) that is the form specifically accounting for most of the unblocked protein chains. This suggests that the free α-amino termini are explained by absence of acetylation rather than by deacetylation. In addition, isozyme II is the predominant form during aeration, when acetyl-CoA might be rapidly removed during efficient cell respiration. It seems possible therefore that both under physiological conditions and in vitro [5], the amount of available acetyl-CoA might be a limiting factor in protein acetylation.

A role of acetylation in protection against protein catabolism is not evident in this yeast system, cf. [3]. During growth to plateau, isozyme I is largely replaced by isozyme II. Even though there is net catabolism of isozyme I during aerobic isozyme changeover, isozyme I is not the form predominantly unblocked. It should be stressed, however, that different types of protein catabolism exist and that long-term effects need not be identical to the factors controlling the inducible changes during aeration in yeast.

The observed lack of effect of acetylation on enzyme activity suggests that there are other functions of acetylation. One new and trivial possibility might even be that it is an additional way of acetyl group

handling in the cell. It would then resemble other α-amino group modifications, involving glucose [18] or CO_2 [19] that reflect high physiological concentrations of these compounds [20].

Finally, there is an interesting parallel between this system and two others in which blocked and unblocked forms have been found. In pig and beef heart malate dehydrogenase, one enzyme is acetylated and the other is free [21], and the same is true for aldehyde dehydrogenase of horse liver [22]. In both cases, the forms differ considerably more than the two yeast isozymes [23] but the interesting point is that in each case the unblocked forms are mitochondrial and the acetylated forms are cytoplasmic. These data reinforce an apparent link between non-acetylation and an environment with efficient aerobic respiration. The yeast alcohol dehydrogenase system has given the first evidence for a consistent variation in acetylation during physiological conditions. It also provides a useful tool for investigating the effect of physiological state on the modification of isozymes, and it may cast some light on protein acetylation in general.

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