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Regulation of NAD^+-Dependent Isocitrate Dehydrogenase in the Citrate Producing Yeast *Yarrowia lipolytica*

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Abstract—The mechanism of the increased accumulation (overproduction) of citric acids in the yeast *Yarrowia lipolytica* while growing in the presence of glucose under nitrogen deficiency was investigated. The limitation of the yeast growth by the source of nitrogen decreases the total content of nucleotides and increases the ratios of ATP/AMP and NADH/NAD^+. NAD^+-Dependent isocitrate dehydrogenase, an enzyme of the tricarboxylic acid cycle playing a key role in the regulation of biosynthesis of citric and isocitric acids, was isolated from *Y. lipolytica*. The molecular weights of the native enzyme and its subunits were found to be 412 and 52 kD, respectively. It is concluded that the enzyme is a homooligomer consisting of eight subunits. Investigation of the effect of some intermediates of the tricarboxylic acid cycle on the activity of this enzyme suggests that the enhanced excretion of citric acids can be caused by the inhibition of NAD^+-dependent isocitrate dehydrogenase due to the decrease in the content of AMP and increase in the NADH/NAD^+ ratio in the cells of *Y. lipolytica* under depletion of nitrogen.

Key words: biosynthesis of citric acids, *Yarrowia lipolytica*, isocitrate dehydrogenase, regulation

Growth limitation of the yeast *Yarrowia lipolytica* by the source of nitrogen with excess carbon source results in intensive synthesis of citric and isocitric acids. After depletion of the nitrogen in the stationary growth phase, the yeast maintains metabolic activity and continues the assimilation of the carbon substrate, accumulating more than 100 g/liter of citric acid in the culture medium [1-3]. In spite of the well-studied microbiological part of the subject [4, 5], biochemical mechanisms of these processes and possibilities of their regulation remain obscure. It is known that the depletion of nitrogen in the medium results in the redistribution of the carbon flows between catabolic and anabolic pathways. The central pathway of carbon metabolism in most living organisms is the tricarboxylic acid (TCA) cycle. The reaction of oxidative decarboxylation of isocitrate catalyzed by NAD^+-dependent isocitrate dehydrogenase (NAD^+-IDH) is suggested to be the initial control point of the cycle, largely determining the rate of the cycle. Adenine and pyridine nucleotides play a significant role in the regulation of cell metabolism and in particular the TCA cycle. In the present article, we investigated changes in the nucleotide pool (ATP, ADP, AMP, NAD^+, and NADH) during the cultivation of *Y. lipolytica* under the conditions of growth limitation by the source of nitrogen. NAD^+-IDH was isolated from *Y. lipolytica*, purified, and partially characterized. Possible mechanisms of regulation of the enzyme and its role in biosynthesis of citric acids are discussed.

MATERIALS AND METHODS

Chemicals. In the present work the following chemicals were used: an HPX-87H ion-exchange column (7.8 × 300 mm; BioRad, USA); a kit of organic acid standards (Sigma, USA); an Octyl-Sepharose column (1.6 × 40 cm), a CL-4B Sepharose column, a 12 HR 1/30 Superose column, and protein standards (Pharmacia, Sweden). Other chemicals were of domestic production or obtained from Sigma.

Object of investigation. The yeast culture *Yarrowia lipolytica* BKM Y-2373 (704) was from the collection of cultures of the Institute of Biochemistry and Physiology of Microorganisms of the Russian Academy of Sciences.

Yeast cultivation. The yeast was grown in mineral Rider medium supplemented by Burkholder's micronutrients [6] and 0.3% yeast extract. Glucose (2%) was used as the carbon source. When cultivating the yeast under

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nitrogen depletion, the content of ammonium sulfate in the medium was lowered to 0.6 g/liter. The yeast was cultivated in ANKUM-2M fermenters (volume 10 liters, working volume 6 liters) at 29°C and 800 rpm, the pH of the medium being adjusted to 6.0 automatically with 10% NaOH. Oxygen content in the medium was maintained at the level of 40-60% saturation and was regulated by the rate of air bubbling.

Biomass monitoring. Content of biomass was determined by weighting.

Content of organic acids was determined using HPLC on an HPX-87H column (7.8 × 300 mm, BioRad) while monitoring the absorption at 210 nm. Organic acids from Sigma were used as the standards. Citric and isocitric acids were not resolved into separate peaks, so they were determined as the sum of the acids.

Extraction of nucleotides. NAD^+ and adenine nucleotides were extracted with acid and NADH with alkali [7]: 7 ml of 0.3 N HCl or 1 N NaOH were added to 20 ml of the cell suspension taken from the fermenter. The suspension was incubated in a thermostat at 50°C for 10 min under constant stirring, and then the extract was cooled and neutralized to pH 7.0. The neutralized extract was centrifuged (10,000*g*, 10 min) and the supernatant was used for the analysis of nucleotides.

Analysis of nucleotides. Adenine nucleotides were determined by the luciferin-luciferase method using an LKB 1251 luminometer (Sweden). AMP and ADP were determined after their conversion into ATP in the presence of corresponding enzymes [8]. Pyridine nucleotides were determined by the cyclic method [9] using a Shimadzu UV-160 spectrophotometer (Japan).

Preparation of cell-free extract. The cells were taken in the beginning of the stationary growth phase, centrifuged (5000*g*, 10 min), washed twice with 0.9% NaCl, resuspended in 50 mM potassium phosphate buffer, pH 7.5, containing 1 mM EDTA, 5 mM DTT, and 0.3 mM phenylmethylsulfonyl fluoride, and broken with Ballotitni glass beads (100-150 μ) using a ball mill (1000 rpm, 3 min). The resulting suspension was centrifuged (10,000*g*, 30 min) to remove unbroken cells and cell fragments. All the procedures were performed at 4°C.

Assaying of enzyme activity. The activities of the enzymes were measured using a double-beam Shimadzu UV-160 spectrophotometer (Japan) at 25°C.

The citrate synthase activity (EC 4.1.3.7) was determined at 412 nm as described in [10]. The reaction mixture contained 100 mM Tris-HCl, pH 8.5, 0.25 mM acetyl-CoA, 0.25 mM oxaloacetate, and 0.1 mM DTNB.

The aconitate hydratase activity (EC 4.2.1.3) was measured at 240 nm in reaction mixture containing 50 mM potassium-phosphate buffer, pH 7.5, and 5 mM isocitrate [11].

The activity of NAD^+-dependent isocitrate dehydrogenase (EC 1.1.1.4) was measured at 340 nm in the standard reaction mixture containing 0.5 mM NAD^+, 1 mM isocitrate, 5 mM $MgCl_2$, 0.5 mM ATP, and 50 mM KH_2PO_4, pH 7.4 [12].

The activity of $NADP^+$-dependent isocitrate dehydrogenase (EC 1.1.1.42) was measured at 340 nm in the standard reaction mixture containing 0.75 mM $NADP^+$, 1 mM isocitrate, 15 mM $MgCl_2$, and 50 mM Tris-HCl, pH 9.0 [12].

The activity of isocitrate lyase (EC 4.1.3.1) was measured at 324 nm. The reaction mixture contained 75 mM potassium-phosphate buffer, pH 6.85, 4 mM isocitrate, 8 mM phenylhydrazine, 4 mM cysteine, and 10 mM $MgCl_2$ [13].

The pyruvate carboxylase activity (EC 6.4.1.1) was determined by measuring the rate of the incorporation of labeled bicarbonate into acid-resistant reaction products [14].

Purification of NAD^+-IDH. All the procedures were performed at 4°C. Ammonium sulfate was added to the cell-free extract to 50% saturation, this resulting in the precipitation of the main part of the enzyme. The precipitate was separated by centrifugation (15,000*g*, 30 min) and dissolved in 10 mM potassium-phosphate buffer, pH 7.5. The pH value of the enzyme solution was adjusted to 3.5 using 10% acetic acid added gradually with constant stirring. The resulting suspension was centrifuged (5000*g*, 10 min). The supernatant free of the NAD^+-IDH activity was discarded, and the precipitate was dissolved in 0.5 M potassium-phosphate buffer, pH 7.5, and centrifuged again (10,000*g*, 10 min) to remove denatured proteins. The subsequent purification included hydrophobic chromatography. Ammonium sulfate was added to the enzyme solution to the final concentration of 2 M. The solution was applied to an Octyl-Sepharose column (1.6 × 40 cm, Pharmacia) equilibrated with 2 M ammonium sulfate in 100 mM potassium-phosphate buffer, pH 7.5. The column was washed with 2 M ammonium sulfate in the buffer until no proteins were detected in the eluate. Then NAD^+-IDH was eluted with 1 M ammonium sulfate solution at 50 ml/h (fraction volume, 12 ml). The fractions containing NAD^+-IDH were pooled and concentrated by adding ammonium sulfate to 60% saturation. The pellet was separated by centrifugation (10,000*g*, 30 min), dissolved in a small volume of 100 mM potassium-phosphate buffer, pH 7.5, and centrifuged repeatedly to remove denatured proteins. The supernatant was applied on a CL-4B Sepharose column (Pharmacia) equilibrated with 100 mM potassium-phosphate buffer, pH 7.5. The enzyme was eluted with the same buffer at 7 ml/h (fraction volume 1.5 ml). The fractions containing the NAD^+-IDH activity were pooled, concentrated, and supplemented with glycerol (final concentration, 20%). This preparation was used in the work.

Determination of molecular weight. The molecular weight of the native preparation of NAD^+-IDH was determined using HPLC on a 12HR 1/30 Superose column (Pharmacia). The enzyme was eluted at 1 ml/min

with 50 mM potassium-phosphate buffer, pH 7.4, containing 200 mM NaCl. Proteins with known molecular weights (ferritin, 440 kD; aldolase, 160 kD; bovine serum albumin (BSA), 67 kD; and cytochrome *c*, 13 kD) (Pharmacia) were used as the standards.

Protein assay. Protein concentration was determined by Bradford's method [15] BSA as the standard. After chromatography, protein concentration in the fractions was determined spectrophotometrically at 280 nm.

Electrophoresis in 12.5% polyacrylamide containing 10% SDS was performed using a Fast-System unit for horizontal electrophoresis (Pharmacia) using standard gels and chemicals for electrophoresis (Pharmacia). Proteins were stained with Coomassie R-250 according to the instruction to the Fast-System unit. A kit of proteins (Bio-Rad) including proteins of known molecular weights was used as the standard: phosphorylase *b* (92.5-97.4 kD), BSA (66-68 kD), ovalbumin (43-45 kD), carboanhydrase (29-30 kD), trypsin (20.1-21 kD), and lysozyme (14.3 kD).

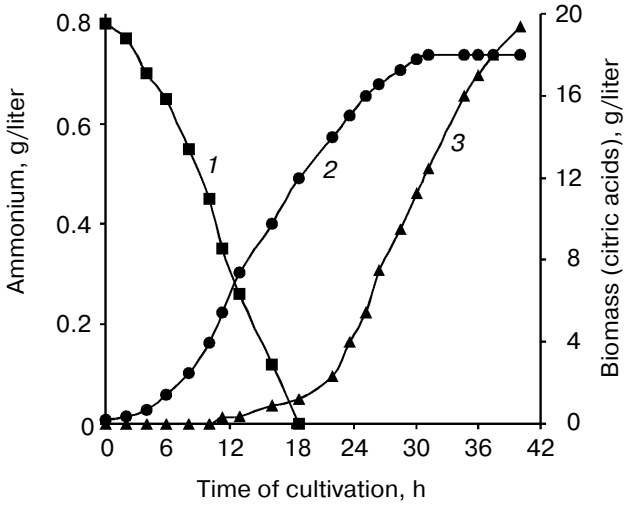

Fig. 1. Dynamics of growth of *Y. lipolytica* and biosynthesis of citric acids in the presence of glucose under nitrogen depletion: *1*) NH_4^+ content; *2*) biomass; *3*) content of citric acids.

RESULTS

Figure 1 presents the growth curve of *Y. lipolytica* during depletion of nitrogen. It is seen that the transition of the culture into the growth deceleration phase (after 19 h) caused by the depletion of nitrogen was accompanied by the accumulation of citric acids in the culture medium. The total concentration of the acids constituted 20 g/liter by the end of fermentation (42 h).

Table 1 presents data on the intracellular content of adenine and pyridine nucleotides during the fermentation. The calculations were made based on the present results and the literature data on the content of the intracellular water in yeast, according to which 1 mg of dry weight of the cells corresponds to 4 µl of water [16]. The transition of the culture into the deceleration growth phase was accompanied by a decrease in the content of each of the nucleotides. The ATP level decreased 3-fold, the level of ADP and AMP decreased sharply, 6- and 10-fold, respectively. The content of NAD^+ in the cells decreased almost 6-fold with the transition of the culture from the active growth to the stationary growth phase. The content of NADH during the active growth decreased to a lesser extent, increasing somewhat in the stationary growth phase.

Table 2 presents the dynamics of the activity of the key enzymes of the TCA cycle in *Y. lipolytica*. The samples for assaying of the enzyme activities were taken in the exponential growth phase (6 h) when no synthesis of the acids was observed, at the deceleration growth phase

Table 1. Concentration of nucleotides in the yeast *Y. lipolytica* under conditions of growth limitation by the source of nitrogen*

Time of cultivation, h	Intracellular concentration of nucleotides, mM				
	ATP	ADP	AMP	NADH	NAD
6	1.85	6.2	3.92	3.88	22.02
19	0.85	1.33	0.96	2.32	9.38
27	0.62	1.02	0.33	1.1	4.76
32				1.86	4.42
40				3.15	3.77

* Extraction of the nucleotides in each sample was performed twice. The resulting extracts were analyzed at least twice. Thus, the presented results are the mean value of the nucleotide concentrations obtained after four measurements. The spread of the measured concentrations was within 15% of the mean values.

Table 2. Activity of the enzymes (μmol/min per mg protein) in the cell-free extracts of *Y. lipolytica* under growth limitation by the source of nitrogen

Enzyme	Growth phase		
	exponential (6 h)	beginning of acid synthesis (19 h)	stationary (27 h)
Citrate synthase	1.3	0.62	0.6
Aconitate hydratase	0.45	0.28	0.3
NAD^+-IDH	0.15	0.17	0.16
$NADP^+$-IDH	0.3	0.26	0.24
Isocitrate lyase	0.03	0.02	0.02
Pyruvate carboxylase	0.048	0.045	0.045

Table 3. Purification of NAD^+-IDH from *Y. lipolytica*

Step	Volume, ml	Protein, mg	Total activity, U	Specific activity, U/mg protein	Yield, %
Cell-free extract	100	760	129.2	0.17	100
$(NH_4)_2SO_4$ (50%)	25	248	104.16	0.42	81
Acidic precipitation	20	40.5	86.27	2.13	67
Octyl-Sepharose	36	12.3	62.12	5.05	48
Gel-filtration	9	1.82	40.05	22	31

Table 4. Effect of metabolites on the activity of NAD^+-IDH

Effector (10 mM)	Activity, % of control
Oxaloacetate	86.5
Citrate	91.1
Succinate	97.2
Fumarate	93.2
Malate	93.2
Glyoxylate	79.0
2-Oxoglutarate	91.3
Glutamate	99.8

(27 h) in the beginning of the synthesis of the acids, and in the beginning of the stationary growth phase (32 h) corresponding to the active synthesis of the acids. In the period of synthesis of the acids, the activity of citrate synthase decreased 2.2-fold, but retained rather high (0.6 μmol/min per mg protein). The activity of aconitate hydratase decreased by 30% and constituted 0.3 μmol/min per mg protein in the stationary growth phase. The activity of NAD^+-IDH remained constant (0.15-0.17 μmol/min per mg protein) during the whole period of culture growth. In the period of active synthesis, the activity of $NADP^+$-dependent isocitrate dehydrogenase decreased by 20%. A low activity of isocitrate lyase suggests the existence of some other anaplerotic pathway of oxaloacetate resynthesis likely including carboxylation of pyruvate. The activity of pyruvate carboxylase did not change during the period of acid synthesis, constituting 0.045-0.048 μmol/min per mg protein.

To investigate fine mechanisms of the regulation of biosynthesis of citric acids, we isolated and partly characterized NAD$^+$-IDH from *Y. lipolytica*. The developed procedure of purification includes four steps (see details in "Materials and Methods"). As seen from Table 3, after the first two steps, a significant amount of ballast proteins was removed (total protein decreased more than 18-fold with the yield of NAD$^+$-IDH 67%). During hydrophobic chromatography, NAD$^+$-IDH bound to octyl-Sepharose at 2 M ammonium sulfate. After washing and eluting of unbound proteins, the enzyme was eluted with 1 M ammonium sulfate solution. Finally, the enzyme was purified by gel filtration on a CL4B Sepharose column. As a result, NAD$^+$-IDH was purified 129-fold with the yield of 31%, the specific activity constituting 22 μmol/min per mg protein. The enzyme was purified to homogeneity, this being confirmed by the results of SDS-PAGE (Fig. 2).

The molecular weight of the native NAD$^+$-IDH determined by HPLC on a 12HR-30 Superose column was 412 kD. Additional experiments on the determination of the molecular weight on a Sepharose 4B column resulted in a similar value (400 kD). The subunit composition of the enzyme was determined by SDS-PAGE. As seen from Fig. 2, a single protein band was observed corresponding to 52 kD, this indicating that NAD$^+$-IDH from the *Y. lipolytica* yeast is an octamer composed of identical subunits.

The activity of the enzyme was maximal at pH 7.4. A shift to more acid or basic media resulted in its rapid inactivation.

To reveal possible regulators of NAD$^+$-IDH, the effect of a number of intermediates of the TCA cycle was investigated. The results presented in Table 4 indicated that all listed compounds little affected the activity of NAD$^+$-IDH.

The K_m value for NAD$^+$ was 136 μM, and the Hill coefficient was 1.06.

Yeast NAD$^+$-IDH exhibits a sigmoid dependence of the activity on isocitrate concentration. It is known that AMP is a positive effector of NAD$^+$-IDH. The dependence of the enzyme activity on isocitrate concentration in the reaction mixture was measured in the presence of different concentration of AMP (Fig. 3). To calculate the kinetic parameters, we used the kinetic model described by the Webb equation [17].

As seen from Fig. 3a, the K_m value for isocitrate in the absence of AMP was 581 μM. AMP increased the affinity to the substrate ($\alpha < 1$), but did not affect the maximal velocity of the reaction ($\beta \approx 1$). NAD$^+$-IDH exhibits a low affinity to AMP ($K_m = 995$ μM). The values of the Hill coefficients suggest the presence of four sites of isocitrate binding and two sites of AMP binding in the enzyme molecule (Fig. 3a). The addition of ATP (0.25 mM) to the reaction mixture resulted in slight changes in the kinetic parameters of the enzyme (Fig. 3b). Additional investigations showed that 0.5 mM ATP did not affect significantly the activity of the enzyme.

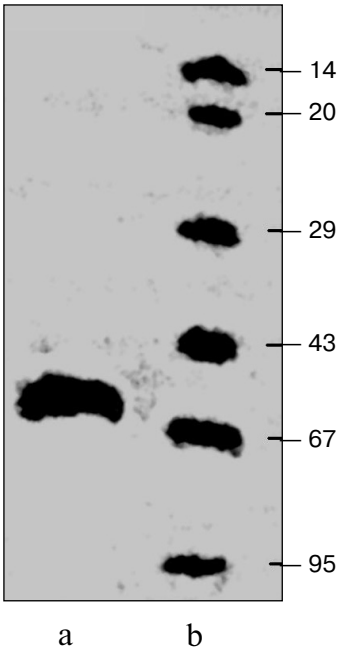

Fig. 2. SDS PAGE of purified NAD$^+$-IDH: a) purified preparation of NAD$^+$-IDH; b) protein standards.

NADH was a strong inhibitor of the NAD$^+$-IDH from *Y. lipolytica*: 0.1 mM NADH inhibited the enzyme by 50%, and 100% inhibition was observed in the presence of 0.2 mM NADH. NADH decreased the affinity of the enzyme to NAD$^+$, acting as the competitive inhibitor ($\alpha > 1$). The K_m value for NADH was 63 μM.

DISCUSSION

Cultivation of *Y. lipolytica* under conditions of nitrogen depletion results in the accumulation of citric acids in the medium. The assay for the acids allowed determination of only the sum of citric acids (citric and isocitric acids). Previous investigations showed that the given yeast strain produced mainly citric acid when growing in medium containing glucose under nitrogen depletion [4].

Investigation of the intracellular content of ATP, ADP, AMP, NAD$^+$, and NADH in *Y. lipolytica* under the conditions of growth limitation by the source of nitrogen and overproduction of citric acids showed that during the active growth phase, the content of adenine nucleotides and NAD$^+$ sharply decreased. The level of NADH changed little during the whole period of cultivation. A similar decrease in the content of adenine nucleotides in the period of the exponential growth was observed in the fungi *Neurospora crassa* [18]. The decrease in the ATP level can be due to the enhanced consumption of energy

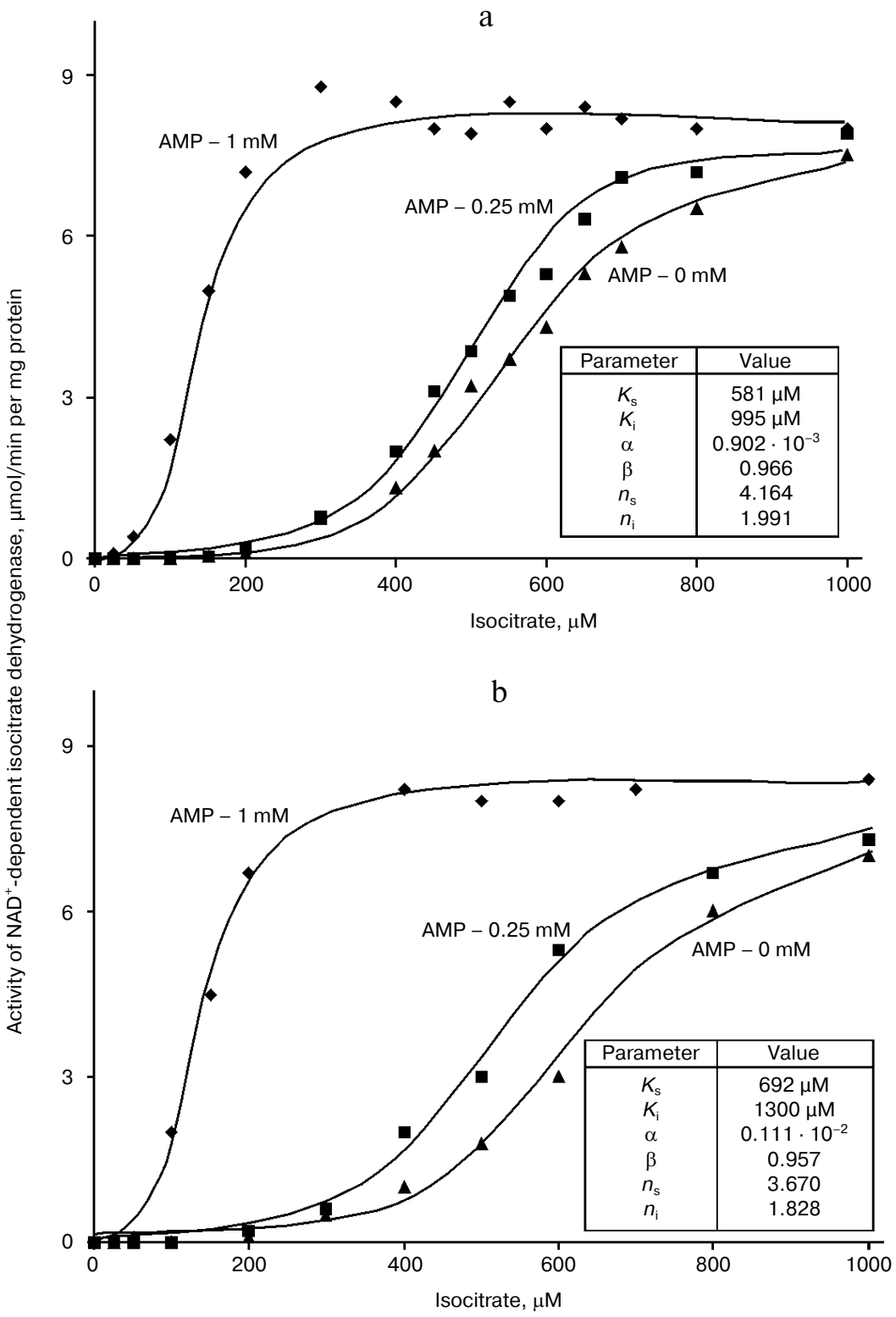

Fig. 3. Effect of AMP on the kinetic parameters of NAD^+-IDH in the absence (a) or in the presence (b) of ATP. The reaction mixture contained 100 mM glycyl-glycine, pH 7.6, 5 mM $MgCl_2$, 1 mM NAD^+, and 0.25 mM ATP. *1*) 1 mM AMP; *2*) 0.25 mM AMP; *3*) in the absence of AMP. K_s is the constant of substrate binding, K_i is the constant of the effector binding, α is the coefficient of the change in K_s in the presence of the effector, β is the coefficient of the change in k in the presence of the effector, n_s is the Hill coefficient for the substrate, and n_i is the Hill coefficient for the effector.

for biosynthesis during active growth. The decrease in the level of ADP and AMP can be explained either by the delay in the rate of their synthesis compared to the rate of growth and division of the cells, or by the presence of a regulatory mechanism directed to the maintenance of the energy homeostasis of the cell. The decrease in the level of NAD^+ with the transition of the culture into the growth deceleration phase might be due to a decrease in the oxidative activity of the cells. The transition of the cells into the growth deceleration phase caused by the depletion of nitrogen in the culture medium is connected with deceleration of biosynthetic processes, and the subsequent stationary phase is due to their complete cessation. The changes in the content of adenine nucleotides in the period of the synthesis of citric acids result in a sharp increase in the ATP/AMP ratio, which plays an important regulatory role in the cells of microorganisms [19, 20].

The analysis of the activity of the key enzymes of the TCA cycle showed that their activities changed insignificantly in the period of citric acid synthesis. In spite of the fact that the citrate synthase activity decreases 2-fold in the phase of acid formation, its level exceeds the activity of the succeeding enzymes of the cycle. This suggests a presence of fine regulatory mechanisms of the overproduction of citric acids. One of the possible points of such a regulation could be the conversion of isocitrate to α-oxoglutarate catalyzed by NAD^+-IDH.

NAD^+-IDH from *Y. lipolytica* was isolated, purified, and partly characterized. The specific activity of the homogeneous enzyme preparation was 22 μmol/min per mg protein. NAD^+-IDH from *Y. lipolytica* is an enzyme of 400 kD consisting of eight subunits of equal molecular weight. The previously studied NAD^+-IDH from baker's yeast [21] and from the yeast *Rhodosporidium toruloides* [22] are also octamers.

We demonstrated that the key intermediates of the TCA cycle at concentrations of 10 mM did not affect significantly the activity of NAD^+-IDH from *Y. lipolytica*. Accumulation of large amounts of citric acids can result in the inhibition of NAD^+-IDH by citrate. In the present work, citrate little affected the purified preparation of the enzyme. However, Sokolov et al. demonstrated that 40 mM citrate inhibited the activity of the partially purified NAD^+-IDH from *Y. lipolytica* by 80% [23]. Considering the complicated interaction of the yeast NAD^+-IDHs with citrate [24-26], the problem concerning the role of citrate in the regulation of the enzyme activity needs special investigation.

Kornberg and Pricer found that NAD^+-IDH from yeast requires AMP [12]. Further investigations demonstrated that NAD^+-IDH possesses an allosteric site for AMP binding, and the interaction of AMP with this site increases the affinity of the enzyme to isocitrate [22, 24, 27]. For NAD^+-IDH from *Y. lipolytica*, AMP was also found to be a positive effector. The results of the kinetic analysis indicate that the enzyme has two sites of AMP binding. The previous studies on NAD^+-IDH from *Saccharomyces cerevisiae* showed that the enzyme consisted of two types of subunits, IDH1 and IDH2, each molecule of the enzyme containing four subunits of each type. The allosteric regulation of the enzyme proceeds due to the binding of AMP to IDH1 [28].

According to literature data, ATP is a strong inhibitor of yeast NAD^+-IDH (even at saturating concentrations of AMP and isocitrate) [12, 22]. The previous work performed on the partially purified enzyme from *Y. lipolytica* also demonstrated the inhibitory effect of ATP on NAD^+-IDH [23]. However, in the present work, we used ATP concentration (10 mM) that significantly exceeded the physiological concentrations of ATP (Table 1) calculated for the cells *Y. lipolytica* based on the experimental data. In the present investigation on the purified NAD^+-IDH from *Y. lipolytica*, the inhibitory effect of ATP was not observed.

Previous works have demonstrated that NADH is an important regulator of the activity of the yeast NAD^+-IDH. The inhibition of the enzyme by NADH was shown for all investigated yeasts [21-23]. The inhibitory effect of NADH on NAD^+-dependent dehydrogenases is a widespread phenomenon. The inhibition is due to the decrease in the dissociation rate of the enzyme–NADH complex, this being determined by the NAD^+/NADH ratio in the cell.

Based on the results on the content of adenine and pyridine nucleotides in *Y. lipolytica* under conditions of growth limitation by the source of nitrogen, as well as the data on the regulation of NAD^+-IDH, we suggest the following mechanism of overproduction of citric acids. The limitation of the growth results in the cessation of biosynthesis of nitrogen-containing compounds (proteins, nucleotides) and reduces their content in the cell. At the same time, the ratios ATP/ADP and NADH/NAD^+ increase, this indicating an enhanced supply of energy and reducing equivalents. This results in blocking of the TCA cycle playing a central role in cell metabolism at the level of NAD^+-IDH. The initial factor that determines the decrease in the activity of the enzyme is the reduced content of the allosteric activator AMP. The subsequent accumulation of citrate can remove this effect. However, further increase in the NADH/NAD^+ ratio enhances the inhibition of NAD^+-IDH, this increasing the pool of isocitrate in the cells. As a result, the equilibrium of the aconitase reaction shifts and the excessive citric acids are excreted into the culture liquid.

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