Vitamin B₆ modulates expression of albumin gene by inactivating tissue-specific DNA-binding protein in rat liver

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The level of albumin mRNA in the liver of vitamin B₆-deficient rats was found to be 7-fold higher than that of control rats. Since the transcriptional activity of the albumin gene, as measured by a nuclear run-on assay, was increased 5-fold in vitamin B₆ deficiency, the higher concentration of albumin mRNA in the liver of vitamin-deficient rats could be attributed to the enhanced rate of transcription. The promoter proximal sequences of the albumin gene interact with a number of tissue-specific transcription factors including HNF-1 and C/EBP. We determined the binding activities of liver nuclear extracts to the HNF-1- and C/EBP-binding sites by gel mobility-shift assay and found that the activities of the extract prepared from liver of vitamin B₆-deficient rats were greater than those of controls. As the

concentrations of C/EBP in nuclear extracts from control and vitamin-deficient rats, estimated by Western-blot analysis, were essentially the same, the lower binding activity of the extract from control liver is probably due to inactivation of tissue-specific factors by pyridoxal phosphate and/or its analogues. We therefore examined the effect of pyridoxal phosphate and its analogues on the binding activity of nuclear extract *in vitro* and found that only pyridoxal phosphate effectively inhibited the binding. These observations indicate that vitamin B₆ modulates albumin gene expression through a novel mechanism that involves inactivation of tissue-specific transcription factors by direct interaction with pyridoxal phosphate.

INTRODUCTION

Vitamin B₆ is an essential water-soluble vitamin required for normal growth and development [1]. The physiologically active form of the vitamin, pyridoxal 5'-phosphate (PLP), is derived from inactive dietary precursors and functions as a cofactor in numerous enzyme reactions of amino acid metabolism [2]. The levels of these enzymes are known to be decreased in tissues of pyridoxine-deficient rats, and high-protein diet aggravates the pyridoxine deficiency [3].

Since Cake et al. [4] presented data that support the concept of PLP as a modulator of rat liver glucocorticoid-receptor interaction, several laboratories have attempted to establish a definitive correlation between vitamin B₆ and steroid-hormone action [5,6].

We have recently found that vitamin B₆ deficiency causes activation of RNA polymerase I and II, and brings about a general enhancement of gene expression in rat liver, including such housekeeping genes as β -actin and glyceraldehyde-3-phosphate dehydrogenase [7,8]. This finding suggests that vitamin B₆ influences gene expression in the liver, at least in part, by modulating the activity of RNA polymerase. However, the magnitude of the increase in albumin mRNA in this study is far greater than can be explained in terms of RNA polymerase activity. In the present study, we examined the effect of vitamin B₆ on albumin gene expression. The albumin gene is expressed in a tissue-specific and temporally regulated manner during mammalian development [9]. Studies of transcription of the mouse and rat albumin genes *in vivo* and *in vitro* have shown that the 170-nucleotide region immediately upstream of the transcription initiation site is sufficient for tissue-specific expression of this

gene [10,11]. Several *cis*-acting elements have been identified in this region that interact with various transcription factors including HNF-1, C/EBP, DBP, CTF/NF1 and NFY [10,11]. The alterations in the transcriptional activity of the albumin gene can therefore be attributed to changes in the availability and/or DNA-binding activity of one or more of these factors. We report here that vitamin B₆ modulates expression of the albumin gene in rat liver through a novel mechanism that involves inactivation of tissue-specific transcription factors.

EXPERIMENTAL

Animals

Male Wistar rats, weighing about 50 g, were given a 70 % casein diet, with or without 0.029 % pyridoxine, *ad libitum* for 4 weeks. The composition of the diet has been described previously [12]. Animals were killed by a blow to the head and decapitated before isolation of the liver. For recovery experiments, rats fed on the pyridoxine-deficient diet received an intraperitoneal injection of pyridoxine (10 mg/100 g body weight) in isotonic 0.9 % NaCl and killed 5 h later.

Determination of PLP concentration

Livers were homogenized with 1 M HClO₄, and a high-speed supernatant was neutralized with KOH to precipitate KClO₄. The PLP content of the resulting supernatant was determined by the apotryptophanase-assay method of Haskell and Snell [13].

Northern-blot hybridization

Total RNA was isolated from the livers and subjected to Northern-blot hybridization using ^{32}P -labelled rat albumin cDNA as described previously [14]. The autoradiographs were densitometrically scanned in order to determine mRNA levels quantitatively.

Nuclear-transcription assay

Nuclei were isolated from the livers and subjected to run-on transcription assay as described by Noguchi et al. [15].

Gel mobility-shift assay

Nuclear extracts were prepared from the livers essentially as described by Dignam et al. [16]. Two oligonucleotides (CTGTAGATCATTAACCA and GTATGTTTGCCATCTGG), representing binding sites of HNF-1 and C/EBP respectively [10], were synthesized on an Applied Biosystems synthesizer, and double-stranded oligonucleotides were labelled with ^{32}P at the 5' end by T_4 polynucleotide kinase. Mobility-shift assay was performed essentially as described by Chodosh [17] in 10 μl containing up to 10 μg of nuclear-extract protein. Nuclear extracts were incubated with poly(dI-dC)·poly(dI-dC) in a binding buffer consisting of 10 mM Hepes (pH 7.5), 50 mM KCl, 2.5 mM MgCl_2 , 0.1 mM EDTA, 1 mM dithiothreitol and 5% glycerol for 30 min at 25 °C, after which ^{32}P -labelled oligonucleotide probes (20000 c.p.m.; ~ 5 fmol) were added and the reaction mixtures incubated for 30 min at 25 °C. In some cases, 50–100-fold concentrations of unlabelled competitor oligonucleotides were added to the reaction mixture. Reaction mixtures were analysed on 5% polyacrylamide gels as described [17].

Western-blot analysis

Nuclear extracts (20 μg of protein) were subjected to PAGE [18]. After electrophoresis, the gel was blotted on to a nitrocellulose membrane and Western-blot analysis was carried out using polyclonal antibody against C/EBP (Santa Cruz Biotechnology, Santa Cruz, CA, U.S.A.).

RESULTS

Increase in albumin mRNA in the liver of vitamin B_6 -deficient rats

Rats were fed on a 70% casein diet, with or without pyridoxine *ad libitum* for 4 weeks. The growth curves of rats in each group

Figure 1 Growth of rats on different diets

■, 70% casein with 0.029% pyridoxine; ●, 70% casein without pyridoxine.

Table 1 Effect of vitamin B_6 deficiency and supplementation on PLP concentration and albumin mRNA level in rat liver

Experimental details are described in the Experimental section. Rats were fed on a diet with (control) or without ($-\text{B}_6$) pyridoxine for 4 weeks. Some vitamin B_6 -depleted rats received an intraperitoneal injection of pyridoxine 5 h before being killed ($-\text{B}_6 + \text{Pyr}$). Northern-blot analysis of albumin mRNA was performed as described in the legend to Figure 2, and the areas under the absorbance peaks corresponding to the mRNA bands were calculated and expressed in arbitrary units. Data are given as the means $\pm$ S.E.M. of four separate determinations.

	PLP concentration (nmol/g of liver)	Relative level of albumin mRNA (arbitrary units)
Control	10.6 $\pm$ 4.1	1.00 $\pm$ 0.06
$-\text{B}_6$	3.3 $\pm$ 1.7	7.05 $\pm$ 2.26
$-\text{B}_6 + \text{Pyr}$	14.3 $\pm$ 2.5	3.52 $\pm$ 0.68

are given in Figure 1. The weight gains were 7 g/day for the control rats and 3 g/day for the rats on the pyridoxine-deficient diet. The concentration of PLP in the liver of rats fed on the vitamin B_6 -deficient diet was reduced to approx. 30% of that in control rats at the end of the feeding schedule, but was completely restored to normal 5 h after the administration of pyridoxine (Table 1). Total RNA isolated from the livers was subjected to Northern-blot analysis using albumin cDNA probe, and the level of albumin mRNA was determined by densitometric tracing. Albumin mRNA concentration in the liver of vitamin B_6 -deficient rats was 7-fold higher than that in control rats (Figure 2). The increased level was reduced to half within 5 h of administration of 10 mg/ml pyridoxine to the vitamin B_6 -deficient rats (Figure 3 and Table 1).

Elevation of albumin mRNA level is due to increased transcriptional rate

To investigate whether an increase in transcriptional rate might account for the elevated level of albumin mRNA, we performed a nuclear run-on assay. As shown in Figure 4, the rate of transcription of the albumin gene was enhanced 5-fold in vitamin B_6 -deficient rats, indicating that the higher level of albumin mRNA in the liver of vitamin-deficient rats could be due to the enhanced rate of transcription. When α -amanitin, an inhibitor of RNA polymerase II, was added to the reaction mixture (2 $\mu\text{g}/\text{ml}$), transcription of the albumin gene was almost completely inhibited (results not shown), suggesting that the transcripts result from RNA polymerase II activity.

Increase in binding activity of nuclear extract to HNF-1- and C/EBP-binding sites in albumin gene during vitamin B_6 deficiency

There is a hierarchy of importance of the various *cis*-acting factors for tissue-specific albumin gene transcription; the HNF-1- and C/EBP-binding sites activate transcription more strongly than the other sites [11]. We synthesized two oligonucleotides, CTGTAGATCATTAACCA (corresponding to nucleotides -44 to -60 of rat albumin gene) and GTATGTTTGCCATCTGG (nucleotides -151 to -167 of rat albumin gene), which interact with HNF-1 and C/EBP respectively [10]. We then assayed the binding activities of nuclear extracts to each of these oligonucleotides by mobility-shift analysis. Figure 5(a) shows that the binding activity of nuclear extract prepared from the

Figure 2 Effect of vitamin B₆ deficiency on the level of albumin mRNA in rat liver

The procedures for RNA isolation and Northern-blot hybridization are described in the Experimental section. (a) Northern-blot analysis of total RNA from the livers of control and vitamin B₆-deficient (-B₆) rats with albumin cDNA probe. The gel shown is representative of four independent experiments. (b) Ethidium bromide staining of ribosomal RNA as internal control of the amount of RNA loaded.

liver of vitamin B₆-deficient rats (lanes 6–10 and lanes 16–20) to both oligonucleotides was greater than that of the controls (lanes 1–5 and lanes 11–15). The sequence specificity of the binding was verified by the addition of 50–100-fold molar excess of unlabelled competitor oligonucleotides (Figure 5b, lanes 1–3 and lanes 7–9). Both unlabelled competitors blocked the formation of the complex between nuclear extract and test DNA probes. This result indicates that the concentration and/or DNA-binding activity of HNF-1 and C/EBP were elevated in the liver of vitamin B₆-deficient rats.

Concentration of C/EBP is unchanged in vitamin B₆ deficiency

To estimate the relative abundance of C/EBP in nuclear extracts, Western-blot analysis was performed using C/EBP-specific antibody. As illustrated in Figure 6, a major band of approx. 42 kDa and a minor band of 32 kDa were observed. The latter probably represents a truncated form of C/EBP, a result of proteolytic degradation during extraction. The fact that the intensities of the bands with nuclear extracts from control and vitamin-deficient rat livers were essentially the same suggests that the control liver contains almost the same amount of C/EBP as the vitamin-deficient liver. We therefore conclude that the low binding

Figure 3 Time course and dose-response of albumin mRNA expression in the liver after the administration of pyridoxine to vitamin B₆-deficient rats

Northern-blot analysis for albumin mRNA was performed as described in Figure 2, and the areas under the absorbance peaks corresponding to the mRNA bands were calculated as described in Table 1. (a) Time course of albumin mRNA expression in the liver after the intraperitoneal injection of pyridoxine (5 mg) into vitamin B₆-deficient rats. (b) Dose-response of albumin mRNA expression in the liver 5 h after the administration of pyridoxine to vitamin B₆-deficient rats.

Figure 4 Effect of vitamin B₆ deficiency on transcription of albumin gene in rat liver

The procedure for nuclear run-on transcription assay is described in the Experimental section. A pUC18 cDNA is shown as a negative control. The gel shown is representative of four independent experiments.

activity of the nuclear extract from control liver, shown in Figure 5, is probably due to inactivation of tissue-specific transcription factors by PLP or its analogues.

PLP inhibits binding activities of nuclear extract to HNF-1- and C/EBP-binding sites *in vitro*

We then examined the effect of PLP and its analogues on the binding activity of nuclear extract *in vitro*. As seen in Figures 7(a) and 7(b), incubation of nuclear extract from vitamin-deficient liver with PLP brought about a rapid and extensive decrease in the binding of the extract to both oligonucleotides specific for HNF-1 and C/EBP. Figures 7(c) and 7(d) show the effects of different concentrations of PLP and its analogues, pyridoxamine

Figure 5 Enhancement of HNF-1- and C/EBP-binding activities in the liver of vitamin B₆-deficient rats

The procedure for gel mobility-shift assay is described in the Experimental section. (a) Mobility-shift assays of HNF-1-binding (lanes 1–10) and C/EBP-binding (lanes 11–20) proteins in nuclear extract (0–10 µg of protein) from the livers of control and vitamin B₆-deficient (–B₆) rats. (b) Effect of addition of competitor of oligonucleotides. Nuclear extract (10 µg of protein) from vitamin B₆-deficient rat liver was assayed in the presence of 50- and 100-fold molar excess of unlabelled competitor oligonucleotides and the mutant oligonucleotides (mHNF-1; CTGTAGATCACC GGCCA, mC/EBP; GTATGCCCGCCATCTGGG) as a control.

Figure 6 Western-blot analysis of C/EBP in nuclear extracts prepared from the livers of control and vitamin B₆-deficient rats

(a) The procedure for Western-blot analysis is described in the Experimental section. Each lane represents nuclear extract from the liver of an individual rat in the control or vitamin B₆-deficient (–B₆) group. (b) Preimmune serum was used as control.

5'-phosphate, pyridoxal, pyridoxamine and pyridoxine, on the binding activity of nuclear extract. At 1 mM concentration only PLP was effective; the other analogues were not effective at concentrations up to 4 mM. This observation indicates that both a phosphate and an aldehyde group are required for inactivation.

DISCUSSION

In the present study, we observed that albumin mRNA level was increased 7-fold in the liver of vitamin B₆-deficient rats. That this increase is directly caused by a decrease in intracellular concentration of PLP or its analogues, not by secondary alterations of liver metabolism accompanying vitamin B₆ deficiency, is indicated by attenuation of albumin mRNA level by the administration of pyridoxine to vitamin B₆-deficient rats (Figure 3 and Table 1). That the attenuation is only partial (3.5-fold higher than the control 5 h after the vitamin supplementation) is compatible with a rather long half-life (~20 h) of albumin mRNA in rat liver [19,20].

We recently reported that vitamin B₆ deficiency caused activation of RNA polymerase I and II, and brought about a general enhancement of gene expression in rat liver, including such housekeeping genes as β -actin and glyceraldehyde-3-phosphate dehydrogenase [7,8]. These findings suggest that

Figure 7 Effect of PLP and its analogues on the binding activity of nuclear extract to HNF-1- and C/EBP-binding sites *in vitro*

(a) Time course of inactivation of HNF-1-binding activity by PLP; (b) time course of inactivation of C/EBP-binding activity by PLP; (c) effect of PLP, pyridoxamine 5'-phosphate (PMP), pyridoxal (PL), pyridoxamine (PM) and pyridoxine (PN) on HNF-1-binding activity of nuclear extract; (d) effect of PLP and its analogues on C/EBP-binding activity of nuclear extract. The procedure for gel mobility-shift assay is described in the Experimental section. For time-course studies (a and b), nuclear extract (10 μ g of protein) prepared from the liver of vitamin B₆-deficient rats was preincubated in the binding buffer at 25 °C in the absence or presence of 4 mM PLP for the indicated times before the addition of ³²P-labelled oligonucleotide probes. For examination of the effect of PLP analogues (c and d), nuclear extract (10 μ g of protein) prepared from the liver of vitamin B₆-deficient rats was preincubated in the binding buffer for 30 min at 25 °C in the presence of PLP or its analogues at the indicated concentrations.

vitamin B₆ influences gene expression in the liver, at least in part, by modulating the activity of RNA polymerase. However, the magnitude of the increase in albumin mRNA in the present study was far greater than could be explained in terms of RNA polymerase activity.

Expression of the albumin gene is known to be regulated by tissue-specific transcription factors such as HNF-1 and C/EBP [10,11]. We have now found that the binding activity of liver nuclear extract to HNF-1- and C/EBP-binding sites in the albumin gene is enhanced during vitamin B₆ deficiency (Figure 5). As the concentration of C/EBP in the nuclear extract, determined by Western blotting (Figure 6), was unchanged in vitamin B₆ deficiency, we concluded that the lower DNA-binding activity of the nuclear extract from control liver was due to inactivation of C/EBP rather than a decrease in its concentration. Although we have not performed a similar experiment with HNF-1 because of unavailability of HNF-1 specific antibody, we expect that HNF-1 will behave similarly to C/EBP.

It is well known that PLP is the coenzyme of several enzymes of amino acid metabolism. Apart from its role as coenzyme, PLP has proven to be an effective inhibitor of many enzymes that have binding sites for phosphate-containing substrates or

effectors, including RNA polymerase [21,22], reverse transcriptase [23], *Escherichia coli* DNA polymerase I [24] and animal cell DNA polymerase [25,26]. In all these cases, PLP is far more effective than its analogues. It typically interacts with proteins by forming a Schiff base between its aldehyde group and primary amino groups, most commonly the ϵ -amino groups of lysine residues [27]. The interaction observed here of PLP with tissue-specific DNA-binding proteins probably also involves Schiff-base formation. As PLP constitutes the major portion of vitamin B₆ vitamers in rat liver [28], we propose that the intracellular level of PLP modulates the activities of tissue-specific DNA-binding proteins *in vivo*.

The question to what extent does the PLP treatment *in vitro* approximate conditions *in vivo* may be raised. The millimolar concentrations of PLP and its analogues used *in vitro* (Figure 7) are about 100 times the concentrations found *in vivo* (Table 1). These high concentrations were used in order to show that only PLP among the B₆ vitamers tested was effective, but the rapidity of the PLP action on the DNA-binding activity of nuclear extract *in vitro* (Figures 7a and 7b) suggests that PLP may work at a considerably lower concentration. However, investigation of various transcription factors and the site(s) of PLP binding must

await the construction of an assay system using purified preparations of transcription factors.

Recent studies by Allgood and co-workers have shown that vitamin B₆ modulates transcription activation by glucocorticoid receptor [29] as well as other members of the steroid-hormone-receptor superfamily [30]. Specifically, the level of the steroid-hormone-mediated gene expression is reduced under conditions of vitamin increase, and enhanced during vitamin deficiency. Allgood et al. [31] have further reported that modulation by vitamin B₆ of glucocorticoid-receptor-mediated gene expression requires transcription factor NF1 in addition to the glucocorticoid receptor. The data presented here demonstrate that vitamin B₆ modulates albumin gene expression in rat liver by inactivating the DNA-binding activity of tissue-specific transcription factors. It remains to be established whether or not the modulations of gene expression by vitamin B₆ involving steroid-hormone receptors and tissue-specific transcription factors share a similar mechanism.

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